REMARKS

Claims 1, 2, 4, 5, 8, 9, 24, 25, 30, 31, 34, 35, 37-40 and 43 are rejected.

Applicant respectfully requests consideration of the following arguments which applicant believes puts the application in complete condition for allowance.

CLAIM REJECTIONS 35 U.S.C. §103

Claims 1, 2, 4, 5, 8, 9, 24, 25, 30, 31, 34, 35, 37-40 and 43 are rejected under 35 U.S.C. §103(a) as obvious over SU 1685448 in view of U.S. Patent Nos. 5,665,366 to Rawlings; 4,112,121 to Tenta; 5,411,741 to Zaias; and the Burback reference.

The Examiner accepted applicant's arguments filed August 18, 2005 as to why DMSO affects the activity of the enzyme, such that DMSO would materially affect the composition and thus cannot be included in applicants' composition, which uses "consisting essentially of" language.

The Examiner's current position is that the phrase "consisting essentially of" excludes DMSO but is open to include theophylline and lanolin. SU 1685448 teaches a composition containing an enzyme, theophylline, DMSO, lanoline and sunflower oil. SU 1685448 does not render applicant's claims obvious because each of the other components in SU 1685448 materially affects the enzyme.

Like DMSO, applicant respectfully asserts and provides supporting references (copies attached) that theophylline, lanolin, and sunflower oil

materially affects the target area for trypsin's action, specifically, the water content and/or degree of hydration is increased. Low water content in the stratum corneum creates a dry condition that induces scaly skin. As shown by Sato (Journal of Dermatological Science 18 (1998) 163-169) this scarcity of water makes stratum corneum trypsin less active thus perturbing desmosomal degradation (page 168).

Theophylline, lanolin, and sunflower oil increase the hydration state by providing water to the target area, thus removing the inhibitory affect of the dry area. Theophylline as described in U.S. Patent No. 5,290,782 is a known diuretic and vasodilator. Water exits blood vessels and enters the surrounding area. Lanolin and vegetable oils, such as sunflower oil, are known moisturizers. Lanolin, an emollient, has similar properties to petrolatum, an occlusive. As demonstrated by Kligman (Cosmetics and Toiletries 93 (1978) 27-35) lanolin has a moisturizing affect on the skin. As shown in Figure 2 (page 32) three weeks of treatment with lanolin decreased the degree of xerosis in human subjects from a grade of about 3 to a grade of about 0.5. Lanolin's moisturizing properties could be still detected up to 14 days after termination of treatment. Sunflower oil, which is an occlusive such as petrolatum and which hinders water loss from the skin, also aids in reversing the high rates of transepidermal water loss in skin with altered stratum corneum architecture (C. Prottey et al., British Journal of Dermatology 94 (1976) pp. 13-21). The water content of the skin as reflected by transepidermal water loss measurements was returned to normal levels (Table 4).

Thus these three ingredients in SU 1685448 affect the enzyme trypsin by altering the hydration state of the targeted skin area. Applicant's invention does not require and by the "consisting essentially of" language specifically excludes theophylline, lanolin, and sunflower oil, as is required in SU 1685448. For at least these reasons, applicant respectfully submits that applicant's invention is not obvious over SU 1685448, thus because the primary reference fails, the secondary references are not supported.

CONCLUSION

Applicant does not believe that there is any fee due with this submission. However, if any fees are necessary the Commissioner may consider this to be a request for such and charge any required fees to Deposit Account No. 23-3000.

The Examiner is invited to contact applicant's undersigned representative with any issues or questions.

Respectfully submitted,

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Regression method for assessing the efficacy of moisturizers

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Moisturizers are used to combat the signs and symptoms of dry skin. Everyone intuitively understands what dry skin is and what it looks and feels like. Investigators, however, have been singularly unable to offer a definition in explicit physical terms. This reflects ignorance, not scientific cautiousness. We know virtually nothing about the nature of the ordinary types of dry skin—those which recur every winter, or the facial dryness about which most women complain. What is wrong with this skin is entirely a matter of speculation. Current views lack scientific support and are mainly speculations.[1] They derive their vitality by providing a tone of scientific rationality for those who formulate and market moisturizing cream. They are, in fact, products of "creative" advertising. Suffice it to say here that work in our laboratory fails to show that dry skin loses water more rapidly; dry skin has less capacity to bind and hold water; or that dry skin lacks those hygroscopic substances euphemistically called "natural moisturizing factor" (NMF).[2] Indeed, except for the outermost shedding portion of the horny layer, we cannot even say that dry skin is dry; that is, lacks water.

In short, when assessing dry skin states we do not know what to measure. We lack methodologies which can furnish quantitative, objective information. Perhaps the instrument described by Christensen and coworkers for measuring the viscoelastic properties of the outer horny layer comes closest to enabling measurements that are meaningful.[3] The electrohygrometer, however, is hardly feasible for routine use and its ultrasensitivity may be a disadvantage.

A host of in vitro methods have been developed which measure the effects of water and other substances on the mechanical properties of the isolated horny layer. These measurements are usually accurate and show considerable laboratory prowess.[4,5] But their relevance to in vivo conditions is very uncertain and, in general, of little proved value in designing moisturizers.

In characterizing dry skin we must resort to clinical descriptions and find words which are appropriate descriptors. It is important to appreciate at the outset that "dry skin" is not a specific diagnosis; it has about the same semantic sensitivity as the term "rash." Skin becomes obviously "drier" after any acute injury, physical or chemical; dryness is associated with chronic inflammatory scaling diseases such as psoriasis and atopic dermatitis. The prototype of dry skin in dermatology is ichthyosis, a group of hereditary disorders in which the skin exhibits thick, fish-like scales. These have absolutely nothing in common with the dry skin states which are under consideration here. They have a thicker but defective horny layer through which water is more rapidly lost and they generally have microscopic features which permit histologic diagnosis. Agents which have keratolytic effects such as salicylic acid and high (60%) concentrations of propylene glycol are beneficial in ichthyosis, as Baden and Alper have clearly demonstrated.[6] However, I strongly disagree with Baden's assertion that keratolytic gels are beneficial in ordinary dry skin syndromes (winter dryness).[7] They are in fact often harmful and in my view absolutely contraindicated except for truly hyperkeratotic conditions. The same caution applies to 10% concentrations of the alpha-hydroxy acids (lactic, pyruvic, glycolic) recently found by Van Scott and Yu to be corrective in some ichthyotic disorders.[8] It should be noted that when lactic acid is put into moisturizing creams it is always buffered and is largely in the ionized lactate form.

Having stated what we are not discussing, it is necessary now to deal as explicitly as possible with the common dry skin problems for which moisturizers are designed and so widely used. First of all, dry skin syndromes are strongly associated with specific climates. The most important by far is the winter season of northern zones. The critical factor is low relative humidity and especially a decrease in the absolute moisture content of the atmosphere; dew point is a reliable indicator of the amount of water vapor in the air. One should read Gaul and Underwood's account for a thoughtful clinical analysis.[9] Wind and cold are aggravating factors but of lesser significance than lack of moisture in the air; low relative humidities are especially prevalent in overheated homes and buildings (regrettably the American standard). A dry, hot climate as in desert regions constitutes another meteorologic background in which low humidity is apt to provoke dry skin (generally abetted by

excessive sun exposure).[10] It is also worth noting that the face and hands are prime targets for chapping conditions precipitated by cold weather. However, these same areas are also subject to continual assault from other sources. Soaps and cleansers are aggravating in many cases. And age is a strong factor in dry skin syndromes other than chapping. After about age 50, dry skin, especially of the lower legs, is a regular accompaniment of the aging process. With each decade it increases in intensity and often becomes one of the most disagreeable aspects of "old age." Because it often itches it interferes with sleep, provokes scratching and is in general a major nuisance. Again, it is when the windows come down in winter and the relative humidity falls below 40% that dry skin of the elderly becomes a troublesome problem. Finally, the predisposition to dry skin is probably hereditable. A family history is often obtainable though solid epidemiologic data are lacking on this point. Some persons never develop dry skin no matter what the weather.

It is clear by now that we are discussing a melange of skin problems none of which are "pure" in the sense of being provoked by one factor. The manifestations may be different in different body regions. A great majority of women over 30 believe they have dry facial skin though this may not be obvious to the observer. Many adults, especially those who spend much time outdoors, suffer from chapping of the hands and face in winter. Housewives who cannot avoid wet work are particularly vulnerable to hand chapping. For a thorough analysis of the common dry skin problems under discussion, one should read the excellent accounts of Chernosky, a master clinician.[11]

If we try to characterize dry skin in morphologic terms we can identify several features. The first is that the surface is rough and uneven: in profile, it has a very irregular contour. Scaling accounts for this roughness. Horny cells normally separate in small clusters and are more or less invisibly shed. In dry skin, desquamation occurs in the form of thin plates and sheets, usually attached along one edge and uplifted or curled along the free margin. This process may be examined in detail by using the scanning electron microscope on silicone skin replicas.[12]

An object moving over this "dry" surface encounters increased friction. Rough skin catches on clothing, especially coarsely woven garments. Though various devices for measuring friction have been designed, their usefulness in assessing dry skin and its response to treatment is unknown.[13,14] The same can be said for instruments that measure "softness" or scratch resistance. In areas where the horny layer is thick, as on the back of the knuckles, it is easy to appreciate another feature of dry skin, namely, that it is brittle and hard. Middleton and Allen have clearly related the suppleness of the horny layer to temperature and to its water content.[15] At low temperatures and low water content, the horny layer cannot be easily stretched. It is inflexible and inelastic, compromising its ability to conform to body movements. An inelastic skin will crack and fissure. Although most prominent on the backs of the hands, the same process is often evident on the legs. The cracks may cleave the epidermis and be subtended by inflammation and even hemorrhage (erythema craquele). Finally, scaling scatters light and the reflections appear to the eye as whitish or greyish. This is very evident in blacks, many of whom complain of "ashiness" on the legs in winter.

Thus we are dealing with a complex phenomenon comprising various optical, frictional, and mechanical changes. It may not be so surprising, therefore, that objective characterization of dry skin has so far eluded us. Our working hypothesis is that dry skin is a subtle disorder of desquamation. The significant changes are close to the surface. Instead of horny cells being shed in invisible clusters, they separate as flakes or squames. Anatomically, the epidermis seems perfectly normal. There is however some undisclosed functional disturbance which causes the shedding abnormality. Although the manifestations of dry skin are localized to the outermost portion of the stratum corneum, the basic defect must be in the underlying viable epidermis which creates the horny layer.

Various terms are used for the common forms of dry skin: the one most appealing to us because it is so noncommital is xerosis. Other terms with slightly different connotations are asteatosis and "chapping." This brings us to moisturizers and their effects. Scholarly dictionaries do not acknowledge "moisturization" or "moisturizers" as respectable English terms. The venerable term, of course, is "moisten," to make wet or moist, although "moistener" has a less appealing sound than "moisturizer." "Moisturizer" is evidently a neologism of the cosmetic industry and has appealing connotations. However, since we do not know what dry skin really is or what moisturizers do, it is the better part of discretion to define a moisturizer in operational rather than conceptual terms, thus: *A moisturizer is a topically applied substance or product that overcomes the signs and symptoms of dry skin* The skin becomes less scaling and therefore smoother and softer, properties which can be seen and felt.

Investigators have described in vivo models for assessing moisturizers, mostly relying on direct visualization to estimate the degree to which the original dryness is relieved or moderated.[16] Whatever else a moisturizer may be or do, it has to improve dry skin. Some workers have tested the efficacy of moisturizers by hand washing tests in which chapping is induced by five daily soap washings for four days with application of the test substance after each of the first four washings.[17] The stereomicroscope is an invaluable aid to visualization in such tests. This model may be suitable for testing hand creams

for use by housewives whose chapped hands are already damaged by excessive surfactant exposure. However, hand washing tests are essentially prophylactic and may favor creams which can inactivate anionic detergents. The degree to which the results have relevance for assessing moisturizers on native dry skin, say facial dryness or leg dryness, is questionable. For example, Highley and coworkers find mineral oil to be almost as effective as petrolatum. In our studies (see below) these are poles apart.

At present, assessing moisturizers on the face is not feasible. The volunteer's perception of dryness is highly subjective and usually does not correspond to the observer's estimate. Of course with any of these models, useful information can be gained regarding aesthetics, cosmetic acceptability and a host of other attributes which affect the salability of the product. In our studies we take no account of hedonics.

Current study

We have been trying to gain a better understanding of xerotic conditions. This has involved years of laboratory investigation which has on the whole been quite unrewarding and unilluminating. One experimental tactic in gaining insight into an abnormality is to evaluate measures which can correct or even worsen the condition. Hence our interest in moisturizers.

We were confronted with many problems, the first one being a suitable model. Foremost among our requirements was complete surveillance of the treatment program. Next was ready availability and access to volunteers with xerotic conditions. We entirely dismissed aesthetic considerations and relied on trained observers to assess the effects. We had many questions for which cosmetic chemists had no suitable answers. For example, are there real differences in efficacy between the marketed moisturizers? In the din of the marketplace it is hard to differentiate fact from fiction. Do moisturizers alter the abnormal desquamation pattern in some fundamental way or do they merely cover over the condition and temporarily alleviate roughness through a lubricating and emollient action?

The model—general characteristics

After studying the effects of moisturizers on various body regions we finally selected the lower legs as the test site. Winter xerosis is a fairly common problem in our area. Its manifestations are most striking and often most disagreeable on the lower legs. This site is the first to be afflicted in late fall and the last to clear up in spring. Dryness of the legs is easily perceived by sight and feel. The scales tend to be larger here and the surface is correspondingly rougher. Sebaceous secretion is minuscular and there is no interference with natural "emollients" (as on the face, for example, where surface lipids are abundant). The lateral region of the midleg, the territory four inches below the knee and four inches above the ankle is fairly large. Even more importantly, it is anatomically a rather uniform territory. Below this lower boundary there is an increasing gradient of scaliness and dryness reaching a maximum on the dorsum of the foot. Finally, on the legs translocation of materials from one side to the other is far less likely than, say, the face and hands. Because both legs are usually involved to a similar degree, the paired comparison method can be used to great advantage.

Xerosis of the lower legs is far more prevalent and more severe in females. While this may reflect an innate sex difference, this site is less protected than in trouser-wearing males; greater exposure to wind and cold almost certainly contributes to the higher prevalence of xerosis in females. Light-skinned females of Celtic extraction (Irish-Scots-Welsh) are particularly vulnerable to xerosis. In addition to sunburning easily, these individuals have integuments which are generally hyperirritable. They have higher susceptibility to all manners of environmental insults.

Blacks are at least as susceptible as whites; one even gains the impression of higher prevalence but this may simply reflect the fact that the whitish scales are more apparent on a dark background. Most blacks, in fact, complain of "ashiness" in the winter; the use of creams and ointments to combat this appearance is almost universal. Some of the popular remedies are not entirely benign. Carbolated vaseline, for example, contains phenol and may worsen the condition through irritation. We prefer not to use black subjects because of uncertainty regarding the effects of long-term use of ointments of unknown composition.

As mentioned earlier, xerosis is a regular accompaniment of the aging process, again most prominently on the lower legs. Almost everyone over 70 has "dry" skin in winter time. For the first few years of the study we evaluated moisturizers in elderly volunteers. We had set up a laboratory in a Philadelphia institution and thus had access to a large number of aged persons with xerosis, all living under the same conditions. Despite the manifest advantages, we came to understand that this population was unsuitable for discriminating among moisturizers. Good results were the rule for almost every formulation. Sometimes highly effective preparations turned out to be quite ordinary when tested under more demanding conditions. Our aged subjects mainly sat quietly indoors in a steady environment. Wear-off or wash-off of applied materials was minimal. Xerosis of the elderly, therefore, is very easy to treat.

Accordingly, we were obliged to be more realistic and to use active subjects exposed to all the rigors of winter. We recruited white female college students for this purpose. These were paid volunteers.

With our institutionalized aged subjects, applications were made daily, weekends included. Saturday and Sunday treatments were exceed-

ingly difficult with college students and we finally had to omit these. To our surprise and satisfaction, there were unexpected benefits. With weekend lapses it was possible to estimate the persistence of improvement on Monday mornings. This gave an early clue to ultimate performance and actually strengthened the severity of the test. With poor moisturizers the appearance on Monday mornings may be only slightly or no better than originally. Good moisturizers have effects which endure over weekends.

It will become apparent that our appraisal of efficacy has little to do with immediate, cosmetic effects. Any oleaginous material will temporarily lubricate the surface, obliterating the roughness and decreasing the scattering of light so that the skin looks and feels smooth. By this criterion, all emollient creams are virtually equivalent, the difference relating largely to subjective preferences. We looked for more substantive changes, reflecting a true alteration in epidermal physiology. By this criterion moisturizers are a "treatment," rather than a "treat."

The very essence of the regression method entails estimating the persistence of beneficial effects after the applications have been stopped. The relapse time, the time required for the skin to return to its original state of dryness, is the measure of efficacy. Follow-up observations at various intervals enable one to estimate the rate of relapse.

It must be realized forthrightly that the method has important limitations. Failure to appreciate sources of variability may nullify the value of the test. The most influential factor is weather. Xerosis is highly sensitive to meteorologic conditions, improving or worsening rapidly, literally within a day or two with sharp weather changes. Accordingly, tests conducted in spring are often spoiled by a few days of warm, moist weather. Even without treatment, severe xerosis may be rapidly transformed into a mild condition by nice weather. Under these conditions all moisturizers are more efficient. Spring is a favorable time to get good results. One should also resist the temptation to start testing when the first few days of cold weather occur in the fall, falsely heralding the early onset of winter. In Philadelphia, the dry skin season begins in earnest in mid-December and yields to the softness of spring in about mid-April. We consider January, February, and March to be the peak months to put moisturizers to the redoubtable test.

Because of vagaries of weather and other uncontrollable variables, life styles of panelists for example, high repeatability cannot be claimed. Though we use a numerical scoring system, the values may differ when the same materials are evaluated at different times. This variability can be offset to a large extent by including a reference material in every test. The results are then interpreted in relation to the standard rather than by absolute numbers. Selection of the reference standard depends on a number of factors. Its performance must be known by repeated testing previously. The reference may be a material with unexcelled "moisturizing" capacity. Or perhaps it can be the market leader, or merely the old product which one hopes to replace with a new improved version.

If it is understood that the results are comparative and relative, variance problems are lessened. The effect of sudden weather changes is moderated; it becomes possible to obtain useful results in the fall and spring as well as in winter at its worst.

Procedure

A test panel consists of ten white, young-adult females with moderate to severe xerosis. In a standard test, the agents are applied by trained personnel to opposite legs twice daily, mornings and afternoons, for five weekdays for three weeks (5 days of application). Dryness is scored each Monday morning for the first three weeks; the no-treatment interval is from the last application on Friday afternoon to just before the first application on Monday morning. The follow-up period begins on the third Monday morning (three days after the last treatment). The legs are graded again on Thursday (six days after the last application) and again four days later on Monday (ten days after treatment). These intervals provide enough data to discriminate among moisturizers. The follow-up period may be lengthened in the case of highly effective substances with which relapse to the original condition may take two weeks and even longer.

For pilot studies or when it is sufficient to know merely whether A is better than, equal to, or worse than B, it is feasible to shorten the treatment period to two weeks. An abbreviated protocol is quite adequate to discriminate between two materials which differ appreciably in effectiveness. Again, the legs are scored three, six and ten days after the last application.

The xerosis is graded on a four-point scale (0 to 3), the highest value being the most severe. One observer does all the grading, integrating the information derived by feeling and viewing the skin into a global score. To become a reliable grader takes much experience; not all are suited to the job. It would be misleading to contend that the grade can be accurately defined with regard to physical features. Not all grade 3 xerotics are alike. Some have more cracking and scaling than others, or the surface feels very dry in the absence of conspicuous scaling. Persons with deep fissures or inflammatory changes (erythema craquele) are excluded, as are those with chronic dermatoses. Frenetic scratchers with many excoriations are not acceptable. At each observation time the mean scores and standard deviations are calculated.

The reliability of the procedure can be improved by keeping the observers and the subjects blind. In the usual sense this means that the identities of the test agents are not indicated

and are as alike as possible physically. The ideal would be to keep the observer from identifying the subject, allowing him merely to inspect the leg site and nothing else. We have used this method to select monitors for dry skin studies. When a panel of subjects is rotated through several totally "blind" examinations in one session, the reliable observers' grade will not vary very much for the same subject.

Results with the regression method

My experience with this method covers several winter seasons. Many types of substances and products have been examined. Some of the studies are preliminary, it having seemed desirable to explore the subject in breadth rather than depth during this early stage. Nonetheless I know of no studies where comprehensive experience with moisturizers has been summarized for public scrutiny. Considering the importance of moisturizers to most women and to millions of persons with dry skin syndromes, the time is overdue for entering the test results into the public domain for discussion and debate.

Petrolatum

Effect of duration of treatment When it comes to efficacy, petrolatum is the unrivalled moisturizer. No material in our experience exceeds it in relieving ordinary xerosis. Though it is repugnant from the aesthetic viewpoint, it is very useful as a benchmark to the experimenter. Although it is a variable mixture of hydrocarbons, we have obtained the same results time and again no matter what the source or color, yellow, white or red.

We applied petrolatum to one leg of three groups of ten female subjects each. The first group was treated for one week (five weekdays), the second for two and the third for three. The legs were graded weekly in the treatment and the posttreatment periods. The study was carried out in January and the subjects were markedly xerotic. The untreated leg furnished a basis of comparison.

This simple study was quite illuminating (fig. 1). With one week of treatment, xerosis was greatly reduced on the Monday morning reading. But, a week later, no vestige of improvement remained. The effect was temporary. Applications for two weeks produced a greater improvement at the first follow-up period; this time, however, the beneficial effects were discernible for at least two weeks. After three weeks of treatment the signs of xerosis were completely obliterated; however, the really notable finding was that the subjects had still not returned to the original state of dryness three weeks after the end of treatment.

We have established that the turnover or renewal time of the stratum corneum in the leg of young adults is about 20 days. It takes a horny cell almost three weeks to make the transit from the bottom to the top of the horny layer. An entirely new horny layer will have been created during this period. Let us suppose that the test material actually corrects the epidermal abnormality that leads to the abnormal sloughing of horny cells in xerosis. A week of treatment could only have temporary effects, being only about one-third as long as the time required to replace the original horny layer. After two weeks of treatment, however, much of the horny layer would have been sloughed and replaced by a new "healthier" tissue. Accordingly, it might take two weeks or so for xerosis to return to the original level. Three weeks of treatment gives even more enduring effects. Indeed, complete regression to the original state was roughly equivalent to the renewal time of the horny layer, namely about 3 weeks.

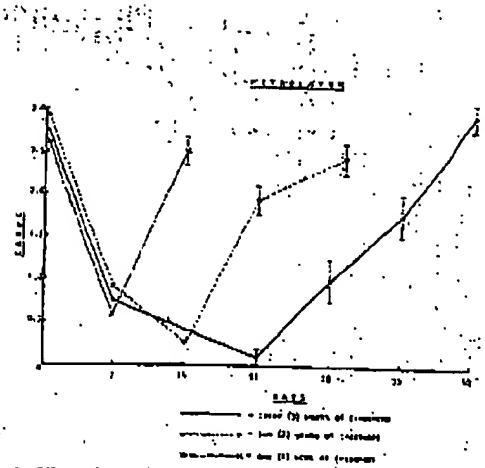

Figure 1. Effect of petrolatum applied one, two and three weeks with follow-ups after one, two and three weeks. One week effects a marked reduction in dryness but the condition rapidly regresses to the original state. After two weeks of treatment, the improvement persists for well beyond a week. Complete relapses may take three weeks after three weeks of treatment.

This is not a suitable place to speculate at length on the mode of action of petrolatum. The effect is not simply due to occlusivity, that is, a reduction in transepidermal water loss. To be sure there is less diffusion of water, but this effect lasts only for some hours after each application. Besides, wrapping the legs for two hours in the morning and again in the afternoon with a completely impermeable plastic film (Saran Wrap) has no beneficial effects whatever. Petrolatum is a tenacious substance which stays in place, filling in the irregularities and smoothing the surface for many hours. Moreover, it is a complex material containing a multitude of chemicals. My own conjecture is that certain of these have pharmacologic effects which can alter epidermal physiology. That some components of petrolatum can penetrate skin is evident by its ability to cause acanthosis (thickening of the epidermis) and to induce comedones. Thus both the physical and pharmacologic properties of petrolatum contribute to its excellence as a moisturizer.

Petrolatum, hydrous lanolin, and o/w emulsions

Effect of frequency of applications For conve-

nience we use twice daily applications. How much more can be expected with more frequent usage? With petrolatums we readily showed that two applications were greatly superior to one in a two week treatment course. Then we determined that thrice daily was slightly more effective than twice. Increasing the frequency of application beyond this had no measurable effect. Six times daily was no better than three. On the other hand, we did not encounter "maceration," though this is often stated, without evidence, to be an untoward result of too frequent use of occlusive ointments.

In another group of ten subjects, we compared hydrous lanolin twice daily to four times daily on opposite legs for three weeks with weekly follow-ups (fig. 2). Four times daily was clearly better than two. It is of more than passing interest that with twice daily applications for three weeks, lanolin was inferior to petrolatum at the first follow-up examination. Moreover, relapse was swifter, with restoration to the original state of xerosis by about two weeks. Nonetheless, lanolin is a quite effective moisturizer and is superior to most moisturizing creams. It is worth noting in passing that we found several brands of "liquid lanolins," especially those miscible with water, to be greatly inferior to lanolin itself.

We conducted similar studies with oil-in-water emulsions containing mineral oil as the major emollient. These are familiar preparations in the marketplace. We generally find that these possess only modest moisturizing powers, the

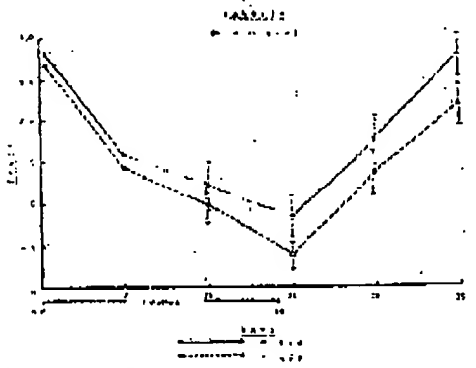

Figure 2. Three weeks of treatment with lanolin comparing two with four applications daily. Four times daily is superior to two and the beneficial effect is more persistent. Lanolin is less effective than petrolatum.

improvement being no longer observable six days after the last application and often gone even after three days. U.S.P. cold cream may be taken as an example and will serve to illustrate the type of results obtained. Using a two week course, four applications daily was greatly superior to two. Two proprietary emulsions behaved similarly. In another group six times daily was compared on opposite sides to four times. The results were slightly better with six times. No difference could be discerned in four of the ten subjects.

It would appear so far that with most emulsion type moisturizers more frequent applications will enhance efficacy. There is a limit to this however. Beyond four times daily, the improvement is generally so slight as not to justify the extra effort. With really poor moisturizers, neat mineral oil for instance, increasing the frequency beyond twice daily has no measurable effect. However, good moisturizing creams become more effective with more frequent use. The enhancement with more abundant use, though obvious, seems not to have been appreciated.

Appraisal of oily substances

Moisturizers must contain oils or oleaginous materials to be effective. Vegetable oils are frequently incorporated in moisturizing creams. Certain of these, olive oil for example, come down from antiquity and are still extensively used in Mediterranean countries. In some hot arid countries corn oil is a favorite, especially among mothers who use it on their children. A variety of vegetable oils are compounded into moisturizing products: sunflower oil, sesame, almond, safflower, and castor. Vegetable oils are essentially mixtures of triglycerides.

Animal fats, also triglycerides, are less admired than formerly but are still to be reckoned with, especially those from novel sources, such as mink and turtle. These appeal to moderns with a strong belief in "natural" products. Dermatologists of a bygone era recommended goose grease for ichthyosis. Lard still has many adherents among dry skin sufferers. Petroleum is of course the chief source of hydrocarbon oils. It is probably safe to say that mineral oil exceeds all other emollients as a major component of moisturizers. We have utilized the regression method to evaluate neat materials of these various classes. The test substance was applied twice daily for three weeks with follow-ups at three, six and ten days. Only one leg was treated.

The results with one oil from each class (mineral oil, goose grease, and olive oil) are shown in figure 3. We note immediately that these are poor moisturizers. Dryness was only slightly moderated at the first follow-up, three days after the last application. In every case the slight benefit achieved regressed within a few days.

More perfunctory studies were done on other oils using two week courses with smaller groups of subjects. The outcomes were equally unimpressive. Crisco (a hydrogenated vegetable oil), turtle oil, almond oil, and sunflower oil behaved about like olive oil.

It cannot be assumed from these results that these oils have no place in moisturizing formulations. It can only be concluded that alone they have small value. Properly compounded, one or more are likely to contribute significantly to a product's moisturizing efficacy. We have found repeatedly, especially with certain lanolin derivatives, that the individual components were comparatively ineffective when evaluated alone.

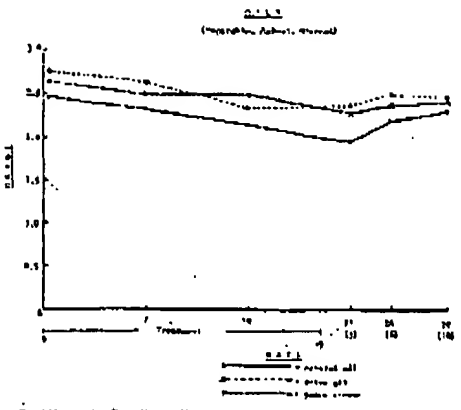

Figure 3. Mineral oil, olive oil and goose grease have minimal moisturizing powers when applied twice daily for three weeks. The figures in parenthesis show the number of elapsed days after the last application.

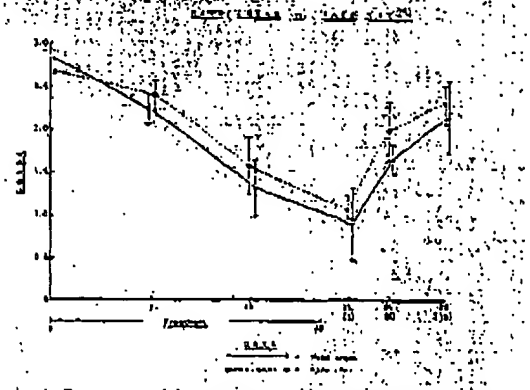

Figure 4. Two successful proprietary products, one a hand-body cream and the other a face cream, show substrate effectiveness. Appreciable improvement persists for 6 days and complete regression has still not occurred after 10 days

but were quite useful when formulated with other agents including waxes, emulsifiers, and stabilizers. For example, we found only modest moisturizing activity with neat isopropyl myristate. Yet this is a valuable ingredient in moisturizing creams, certainly more so than mineral oil. It is the entire mix that counts. The regression method enables the formulator to ascertain the functionality of the various components and to assemble components in such a way as to bestow upon the total composition a degree of efficacy which exceeds the sum of the individual ingredients.

Evaluation of proprietary creams

It seemed highly desirable to gain an idea of the moisturizing efficacy of formulations which had achieved prominence in the marketplace. Obviously we could study only a few of the many available products. We wanted to learn whether there were real differences among commercial moisturizers. We wanted also to know how well these performed in comparison to specified substances such as lanolin. An enterprise such as this has built-in hazards and inevitably raises questions of impartiality. The choice of test materials was arbitrary.

I will invoke the doctrine of discretion as an apology for not revealing brand names. We picked an "elite" facial moisturizer and a hand-body cream formulation. Each has wide acceptance in the marketplace. But more importantly both perform moderately well with the regression procedure and have served as useful benchmarks for assessing other proprietary formulations. As a matter of balance let me say that high sales volume is an unreliable indicator of moisturizing efficacy. This includes so-called market leaders, which sometimes are very ordinary and may even be irritating.

The results obtained with the two proprietary creams on separate groups of ten subjects are portrayed in figure 4. The agents were applied to one leg twice daily for three weeks. There was no statistically significant difference between the two. Both had appreciable moisturizing efficacy and were superior to U.S.P. cold cream but less effective than lanolin. Hydrophilic ointment U.S.P. ranks somewhere between these and U.S.P. cold cream (fig. 5); that is to say, it is modestly active as a moisturizer.

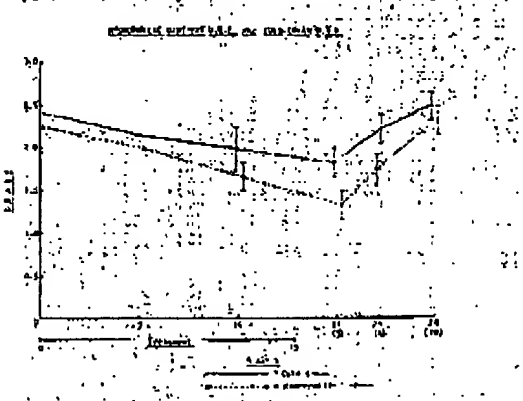

Figure 5. Cold cream, U.S.P., typical of many mineral oil-based moisturizers, has very limited ability to overcome xerosis. Hydrophilic ointment, U.S.P. is superior but is only a fair moisturizer.

It would seem in any case that the regression method has adequate powers of discrimination. Between the most and the least effective formulations, there is a considerable range. This may be a suitable place to present a synopsis of various findings.

- Most dry skin creams based on mineral oil are less effective than U.S.P hydrophilic ointment. They are only fair moisturizers.
- Some moisturizers are available in lotion and cream forms, the former being o/w emulsions of lesser viscosity but greater cosmetic acceptability. We compared Keri Lotion (Westwood) to Keri Cream in a twice daily two week course on opposite legs. The cream was superior, though not strikingly so. In general the greater the concentration of oil, the greater the efficacy. This is indeed the compounder's dilemma: oilier formulations are generally more

effective but less attractive.

• Moisturizers customarily contain humectants, hygroscopic substances which are supposed to bind and retain water. Two of these have received a great deal of attention recently: sodium pyrrolidone carboxylic acid and sodium lactate.[18,19] These are generally incorporated in creams at about 5% concentration. We have taken some pains to conduct paired comparison tests on opposite legs using the same creams with and without these humectants as prepared by the manufacturer. In no instance have we been able to show a beneficial effect from the presence of either of these substances. Both together and each one alone in no way improved the performance of the base in which they were incorporated.

Likewise the addition of 10% propylene glycol, 10% glycerol and 10% sorbitol has given no evidence of improved efficacy when each was incorporated in U.S.P. cold cream.

Urea is a special case in that it has additional properties, such as the ability to disperse proteins. Urea-containing creams are quite popular at present. I have conducted at least a half dozen paired comparison tests with 10%, 20% and even 30% concentration of urea against the base alone. Various market moisturizing creams were utilized in these comparisons. In no instance has it been possible to detect any benefit from the urea. With high concentrations stinging and even mild irritation may develop, especially in subjects with fissures or pruritic skin which has been heavily scratched. There are reports of benefits from urea-containing creams in patients with various types of ichthyosis.[20] In ordinary dry skin syndromes, the use of urea cannot be justified.

Effect of dose

There seems to have been no serious effort to determine whether the effectiveness of moisturizers is dose dependent, and whether increasing the amount applied will increase the beneficial effect. The slope of the dose-response curve may convey important information regarding the potency of the preparation. Will doubling the amount provide a correspondingly large degree of improvement? Then too what is the threshold level, the least amount which will yield a palpable effect? In short, does it make much difference whether one is abstemious or extravagant in using moisturizers?

With sunscreens we have found that the amounts individuals apply to just cover the surface depend very much on the nature of the material. Solutions are likely to be more generously used than emulsions and emulsions more than ointments. Greasier materials are more thinly applied. With U.S.P. cold cream we have found that about 0.50 ml will just cover the lower leg between the knee and the ankle of the average female. Rubbing that amount over this area for 15 seconds leaves a thin film, barely palpable to the subject. On the other hand, 1 ml of that cream is a goodly amount, while 2 ml is greatly in excess of what the most enthusiastic user would ever apply. Using these quantities as rough guidelines we compared various moisturizers on opposite legs, varying only the amounts. The agents were applied twice daily for two weeks with follow-ups at three, six and ten days.

With petrolatum 1.0 ml was questionably better than 0.50. However, 2.0 ml was certainly no better than 1.0 ml. What is the minimal amount that will produce a palpable effect? We massaged 2.0 ml of petrolatum into the area and then removed the excess by thorough wiping with facial tissue. The control leg received 0.5 ml in the usual way. The latter was more beneficial. Nonetheless, the wiped side did improve, perhaps to the degree of a moderately effective moisturizer. Hence, very small amounts of petrolatum produce perceivable improvement.

With hydrophilic ointment U.S.P., 1.0 ml was greatly superior to 0.50 ml. The latter was really ineffective. 2.0 ml in turn was superior to 1.0 ml. In this case, improvement was strongly dose dependent.

With a fair-to-poor moisturizer like U.S.P. cold cream, 1.0 ml was no better than 0.5 ml, neither of which were impressive. With 2.0 ml, effectiveness was clearly enhanced but not greatly. Here, liberal applications added only slightly to efficacy.

With the proprietary facial moisturizer mentioned above, 1.0 ml was superior to 0.50 ml and 2.0 ml was better than 1.0 ml. Here, generous application greatly enhanced effectiveness.

It seems that dosage-response patterns are strongly influenced by the nature of the test material. With heavy ointments, large amounts add little. Liberal application of poor moisturizers does not transform these into effective products. On the other hand, generous use of effective emulsions measurably improves the beneficial effects. In short, the deficiencies inherent in poor creams cannot be overcome by using large amounts while more liberal use of good moisturizers enhances effectiveness.

Comments

It may be useful to summarize the views which have condensed out of this rather desultory foray into the lush field of moisturizers. Our ignorance is still vast and the opinions to be proffered are both personal and tentative. We have a clearer understanding of what moisturizers do not do. Virtually all moisturizing products are emulsions which contain water as a major constituent. But this water evaporates rapidly as the emulsion is rubbed into the skin and has nothing to do with the moisturizing effect. This can be shown by immersing the dry leg in water for five minute periods six times a day at hourly intervals for two weeks. This has no effect whatever in relieving the xerosis. After 10 minute immersions the horny layer will dry out completely in about 20 minutes in heated rooms.

Clearly it is not the function of moisturizers to deliver water to the skin.

Of course, the most effective moisturizers, anhydrous lanolin and petrolatum, have little or no water. Perhaps they "moisturize" indirectly by retarding loss of water? Thick oleaginous substances such as these are said to be occlusive. To be sure, petrolatum in sufficient thickness will prevent diffusional water loss. However, under ambient conditions of use, this effect does not last for more than a few hours. Adding to the considerable natural barrier to water loss provided by the horny layer may contribute to the efficacy of petrolatum. This cannot be the case for lanolin. At normal levels of application (about 2.0 mg/cm^2), the occlusivity of lanolin is slight; the effect is gone within half an hour. Lanolin is rather permeable to water. Thus greasiness is not the whole story. Goose grease is greasy in the extreme, yet it is ineffective as a moisturizer.

My conjecture is that effective greases resist wear-off, reduce friction and roughness, and perhaps most importantly of all contain substances which cross the horny layer barrier, altering the epidermis so that it does not desquamate abnormally when the dew-point is low. I hold that moisturizers have undisclosed pharmacologic effects on epidermal physiology. I shall merely mention here that microscopic examination of skin which has been "cured" of xerosis by petrolatum gives no clue of what has been wrought.

A distinction can be made between immediate and persistent effects. All oleaginous substances are emollients and with frequent application can mask or cover the manifestations of dry skin. No moisturizer can be said to be completely ineffective if one is satisfied with immediate benefits. They all make the skin smoother immediately after application. These temporary effects are the ones which can be easily measured in vitro on the horny layer, a quite dead tissue by the way. Consumers are largely unable to differentiate among the moisturizers which have only temporary masking effects and those which bring about a physiologic change which endures. It can be argued that the distinction is unimportant—relief occurs each time the cream is applied. My bias is that products which correct the basic defect underlying xerosis are in fact more helpful. The merit of the regression method is that it permits, in a moderately objective way, the recognition of moisturizers with true biologic effects. It provides a more reliable means of discriminating among moisturizers as compared to test marketing. Subjective responses are replaced by objective observations in an experimental, controlled setting. Though the effects are judged clinically rather than by specific measurements, means are afforded for empirically formulating better moisturizers. The creation of a moisturizer is still an art, a trial and error endeavor, resting on a weak scientific foundation. One cannot sit at a desk and compose a superior moisturizer on rational principles unless of course aesthetics are ignored. In that case the problem is solved. Petrolatum is matchless!

On the other hand, the regression method does enable one to assess the contribution made by the individual components of moisturizers; many of these contain more than a dozen ingredients. There can be little doubt that the constituents are often chosen to suit popular prejudices or to satisfy the fantasies of marketing men. Is the inclusion of vitamins A, C and D justified? I would wager that their removal would not matter at all. Likewise, the emphasis on the "natural moisturizing factor" (N.M.F.) has produced patents, publicity and products, everything but proof of efficacy.

While we lack the intelligence to formulate moisturizers rationally, the least that can be said for the regression method is that it helps to discard the irrational.

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United States Patent [19]

Suzuki et al.

[11] Patent Number: 5,290,782

[45] Date of Patent: Mar. 1, 1994

[54] XANTHINE DERIVATIVES

[75] Inventors: Fumio Suzuki, Mishima; Junichi Shimada; Akio Ishii, both of Shizuoka; Tetsuji Ohno, Shizuoka, all of Japan; Akira Kurusawa, Huntingdon Valley, Pa.; Kazuhiro Kubo; Hiromi Nonaka, both of Shizuoka, Japan

[73] Assignee: Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

[21] Appl. No.: 839,690

[22] Filed: Feb. 24, 1992

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 574,447, Aug. 29, 1990, abandoned.

[30] Foreign Application Priority Data

Sep. 1, 1989 [JP] Japan 1-226642
Feb. 25, 1991 [JP] Japan 3-029796

[51] Int. Cl.[5] C07D 473/02; A61K 31/52

[52] U.S. Cl. 514/263; 544/225; 544/226; 544/273

[58] Field of Search 544/225, 226, 273; 514/263

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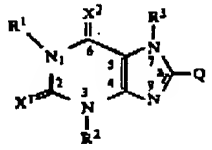
Primary Examiner—Marianne M. Cintins
Assistant Examiner—Catherine Scalzo
Attorney, Agent, or Firm—Fitzpatrick, Cella, Harper & Scinto

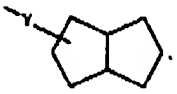
[57] ABSTRACT

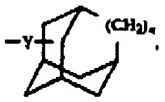
Novel xanthine compounds represented by the following formula:

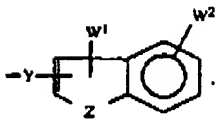


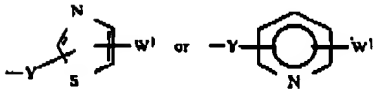
wherein each of X^1 and X^2 independently represents oxygen or sulfur; and Q represents;

(Abstract continued on next page.)

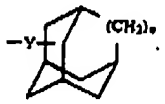




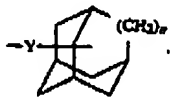




where —— represents a single bond or a double bond; Y represents a single bond or alkylene, n represents 0 or 1, each of W^1 and W^2 independently represents hydrogen, lower alkyl or amino, Z represents $—CH_2—$, —O—, —S— or —NH—; represents

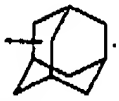


each of R^1 and R^2 independently represents hydrogen, lower alkyl, allyl or propargyl; and R^3 represents hydrogen or lower alkyl, and when Q represents the groups other than



each of R^1, R^2 and R^3 independently represents hydrogen or lower alkyl;

provided that when Q is



then R^1, R^2 and R^3 are not

simultaneously methyl; and pharmaceutically acceptable salts thereof have a diuretic effect, a renal-protecting effect and a bronchodilatory effect.

11 Claims, No Drawings

XANTHINE DERIVATIVES

This application is a continuation-in-part of application Ser. No. 574,447, filed Aug. 29, 1990, now abandoned.

BACKGROUND OF THE INVENTION

The present invention relates to novel xanthine compounds having a diuretic effect, a renal-protecting effect and a bronchodilatory effect.

Heretofore, theophylline, i.e., 1,3-dimethylxanthine has been known as a diuretic, a vasodilator, etc. [The Merck Index, 10th edition, 9110 (1983)].

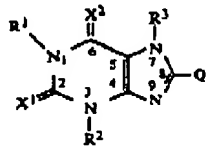
Xanthine compounds carrying, at the 8-position thereof, substituents such as alkyl, alicyclic alkyl, aralkyl, aryl, etc. have a diuretic effect, as disclosed in East German Patent No. 31,772 [Chem. Abst., 63, 18120d (1965)]and West German Patent No. 1,245,969 [Chem. Abst., 67, 90994n (1967)].

In relation to the compounds of the present invention, 8-(1-adamantyl)-1,3,7-trimethylxanthine is described in Tetrahedron Lett., 27, 6337 (1986). However, nothing is mentioned on its pharmacological effect. Further, 8-(1-adamantyl)-1,3-dipropylxanthine having an activity of antagonizing adenosine A_1 receptor is described in J. Med. Chem., 33, 1906 (1990).

The object of the present invention is to provide novel xanthine compounds exhibiting strong diuretic and renal-protecting effect, based on the finding that xanthine compounds which are adenosine receptor antagonists, particularly those having an activity of selectively antagonizing adenosine A1 receptor, have strong diuretic and renal-protecting effect.

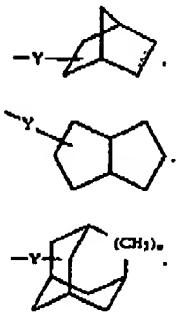
SUMMARY OF THE INVENTION

The present invention relates to a xanthine compound represented by the following formula (I):



(I)

wherein each of X^1 and X^2 independently represents oxygen or sulfur; and Q represents:



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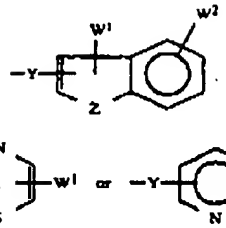
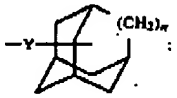
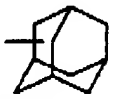


where —— represents a single bond or a double bond; Y represents a single bond or alkylene, n represents 0 or 1, each of W^1 and W^2 independently represents hydrogen, lower alkyl or amino, Z represents $-CH_2-$, —O—, —S— or —NH—; such that when Q represents

each of R^1 and R^2 independently represents hydrogen, lower alkyl, allyl or propargyl, and R^3 represents hydrogen or lower alkyl, and when Q represents groups other than



each of R^1, R^2 and R^3 independently represents hydrogen or lower alkyl;
provided that when Q is



then R^1, R^2 and R^3 are not simultaneously methyl; referred to as "Compound (I)" and compounds with other formula numbers are likewise referred to], or a pharmaceutically acceptable salt thereof.

DETAILED DESCRIPTION OF THE INVENTION

In the definition of the respective groups in the formula (I), the lower alkyl includes straight or branched alkyl having 1 to 6 carbon atoms, for example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl, hexyl, etc The alkylene includes straight or branched alkylene having 1 to 4 carbon atoms, for example, methylene, ethylene, trimethylene, tetramethylene, methylmethylene, propylene, ethylethylene, etc.

The pharmaceutically acceptable salt of Compound (I) includes pharmaceutically acceptable acid addition salt, metal salt, ammonium salt, organic amine addition salt, amino acid addition salt, etc.

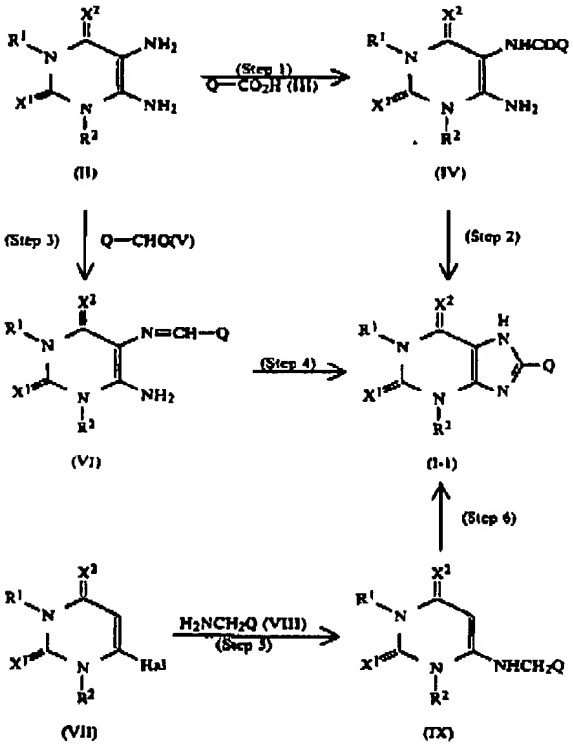
The pharmaceutically acceptable acid addition salt of Compound (I) includes inorganic acid salt such as hydrochloride, sulfate, phosphate, etc., and organic acid salt such as acetate, maleate, fumarate, oxalate, citrate, etc.

The pharmaceutically acceptable metal salt includes alkali metal salt such as sodium salt, potassium salt, etc., alkaline earth metal salt such as magnesium salt, calcium salt, etc., and also aluminum and zinc salts.

The pharmaceutically acceptable ammonium salt includes salts of ammonium, tetramethylammonium, etc. The pharmaceutically acceptable organic amine addition salt includes addition salts of morpholine, piperidine, etc., and the pharmaceutically acceptable amino acid addition salt includes addition salts of lysine, glycine, phenylalanine, etc.

A process for producing Compound (I) of the present invention is described below.

Compound (I-1) which is Compound (I) wherein R^3 is hydrogen, is produced by the following production steps:



wherein Hal represents halogen such as chlorine, bromine or iodine and R^1, R^2, X^1, X^2 and Q have the same meanings as defined above.

Step 1

A Compound (IV) can be obtained by reacting a uracil derivative (II) obtained according to a well known process [for example, the process disclosed in Japanese Published Unexamined Patent Application No. 42383/84] with carboxylic acid (III) or a carboxylic acid reactive derivative.

The carboxylic acid reactive derivative includes acid halides such as acid chlorides, acid bromides, etc., active esters such as p-nitrophenyl ester, N-oxysuccinimide ester, etc., acid anhydrides commercially available or those formed from carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, diisopropylcarbodiimide, dicyclohexylcarbodiimide, etc.; mixed acid anhydrides with monoethyl carbonate, monoisobutyl carbonate, etc. and so forth.

The reaction of Compound (II) with Compound (III) is carried out without any solvent at a temperature of 50° to 200° C. In the case of using the carboxylic acid reactive derivative, the reaction can be carried out according to a process usually used in the peptide chemistry. For example, the reaction solvent is properly selected from halogenohydrocarbons such as methylene chloride, chloroform, dichloroethane, etc., ethers such as dioxane, tetrahydrofuran, etc., dimethylformamide and dimethylsulfoxide, and if necessary water is used. The reaction temperature is -80° to 50° C., and the reaction is completed for 0.5 to 24 hours. Sometimes, the reaction may be favorably carried out, if necessary, in the presence of an additive such as 1-hydroxybenzotriazole, etc., or a base such as pyridine, triethylamine, dimethylaminopyridine, N-methylmorpholine, etc. Furthermore, the carboxylic acid reactive derivative may be formed in the reaction system and used without isolation.

Step 2

A desired Compound (I-1) is obtained from Compound (IV) by the reaction in the presence of a base (process A), by treatment with a dehydrating agent (process B), or by heating (process C).

As the preferable base in the process A, alkali metal hydroxides such as sodium hydroxide, potassium hydroxide, etc. can be exemplified. As the reaction solvent, water, lower alcohols such as methanol, ethanol, etc., ethers such as dioxane, tetrahydrofuran, etc., dimethylformamide, dimethylsulfoxide, etc. can be used alone or in combination. The reaction is carried out at a temperature of from room temperature to 180° C. and is usually completed for 10 minutes to 6 hours.

As the dehydrating agent for use in the process B, thionyl halides such as thionyl chloride, etc., and phosphorus oxyhalides such as phosphorus oxychloride, etc. can be used, and the reaction is carried out at a temperature of from room temperature to 180° C. without any solvent or in a solvent inert to the reaction, for example, halogenohydrocarbons such as methylene chloride, chloroform, dichloroethane, etc., dimethylformamide, dimethylsulfoxide, etc. and is usually completed for 0.5 to 12 hours.

In the case of process C, the Compound (I-1) can be obtained by heating Compound (IV) at a temperature of 50° to 200° C. in a polar solvent such as dimethylsulfoxide, dimethylformamide Dowthermo A (product of Muromachi Kagaku Kogyo Kaisha, Ltd.), etc.

Step 3

A schiff base (VI) can be obtained by reacting Compound (II) with aldehyde (V) in a mixed solvent such as a mixture of acetic acid with a lower alcohol such as methanol, ethanol, etc. at a temperature of -20° to 100° C.

Step 4

A desired Compound (I-1) can be obtained by subjecting Compound (VI) to an oxidative cyclization reaction.

As the appropriate oxidizing agent, oxygen, ferric chloride, ceriumIV ammonium nitrate, diethyl azodicarboxylate, etc. can be exemplified. The reaction is carried out by heating Compound (VI) at from room temperature to 180° C. in the presence of the afore-mentioned oxidizing agent and, if necessary, in a solvent inert to the reaction, for example, a lower alcohol such as methanol, ethanol, etc., a halogenohydrocarbon such as methylene chloride, chloroform, etc., or an aromatic hydrocarbon such as toluene, xylene, nitrobenzene, etc.

Step 5

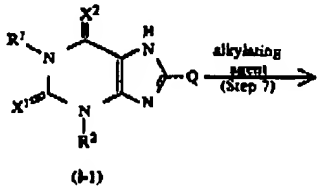
A Compound (IX) can be obtained by reacting a uracil derivative (VII) obtained according to a well known process, for example, the process described in Japanese Published Unexamined Patent Application No. 5082/86 with an amine (VIII) in a solvent inert to the reaction, for example, a lower alcohol such as methanol, ethanol, etc., dimethylformamide, dimethylsulfoxide, etc. alone or in combination thereof at a temperature of 50° to 150° C.

Step 6

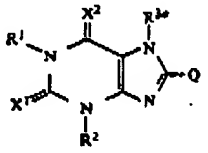
A Compound (I-1) can be obtained by reacting a Compound (IX) with a nitrosating agent such as sodium nitrite, isoamyl nitrite, etc. under an acidic condition with dilute hydrochloric acid, etc. in a solvent inert to the reaction, for example, a lower alcohol such as methanol, ethanol, etc. usually at a temperature of from room temperature to the boiling point of the solvent.

Step 7

A Compound (I-2) which is Compound (I) wherein R^3 is a lower alkyl group can be obtained through the following step:



(I-1)



(I-2)

wherein R^1, R^2, X^1, X^2 and Q have the same meanings as defined above, R^{3a} represents lower alkyl in the definition of R^3.

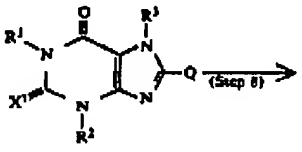
A desired compound (I-2) can be obtained by reacting Compound (I-1) obtained in Steps 1 to 6 with an alkylating agent preferably in the presence of a base.

As the alkylating agent, alkyl halides, dialkyl sulfates, diazoalkanes, etc. are used.

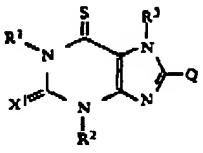
As the base, an alkali metal carbonate such as sodium carbonate, potassium carbonate, etc., an alkali metal hydride such as sodium hydride, etc., and an alkali metal alkoxide such as sodium methoxide, sodium ethoxide, etc. are exemplified. The reaction is completed at a temperature of 0° to 180° C. usually for 0.5 to 24 hours.

Step 8

Compound (I-4) which is Compound (I) wherein X^2 is sulfur, can be obtained by the following step.



(I-3)



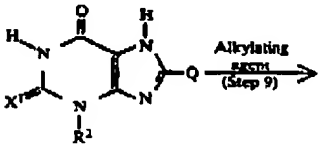
(I-4)

wherein R^1, R^2, R^3, X^1 and Q have the same meanings as previously defined.

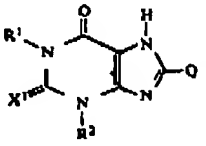
A desired Compound (I-4) was prepared by reacting Compound (I-3) which is Compound (I) wherein X^2 is oxygen, with an appropriate thionation reagent, in an inert solvent. As the thionation reagent, phosphorus pentasulfide and the like are mentioned. As the solvent, dimethylformamide, tetrahydrofuran, dioxane, etc. are mentioned, and preferably pyridine and the like are used. The reaction is carried out at a temperature of 50° to 180° C. for a period of 10 minutes to 36 hours.

Step 9

Compound (I-5) which is Compound (I) wherein R^3 is hydrogen and X^2 is oxygen, can be obtained by the following step.



(X)



(I-5)

wherein R^1, R^2, X^1 and Q have the same meanings as previously defined.

Compound (I-5) can be obtained by reacting Compound (X) obtained according to a similar procedure to Steps 1 to 6 with an equimolar amount of an alkylating agent, if necessary, in the presence of a base.

As the alkylating agent, alkyl halides such as alkyl bromide, propargyl bromide, etc., sulfonic acid esters such as propargyl p-toluenesulfonate, allyl methanesulfonate, etc. are used.

As the base, an alkali metal carbonate such as sodium carbonate, poassium carbonate, etc., an alkali metal hydride such as sodium hydride, etc., and an alkali metal alkoxide such as sodium methoxide, sodium ethoxide, etc. are exemplified. As the reaction solvent, lower alcohols such as methanol, ethanol, etc., ethers such as dioxane, tetrahydrofuran, etc., dimethylformamide, dimethylsulfoxide, etc. can be used alone or in combination. The reaction is carried out at a temperature of from room temperature to 180° C. and usually completed in from 10 minutes to 6 hours.

The intermediates and the desired compound obtained according to the aforementioned processes can be isolated and purified by subjecting them to a purification process usually used in the organic synthetic chemistry, for example, filtration, extraction, washing, drying, concentration, recrystallization, various chromatographies, etc. The intermediates can be used in the successive reaction without any purification.

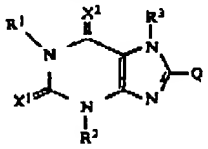
Salts of Compound (I) can be obtained by direct purification when Compound (I) can be obtained in a salt form, or by formation of a salt according to a usual procedure when the Compound (I) is obtained in a free form, and a subsequent purification.

Compound (I) and its pharmaceutically acceptable salts sometimes exist in an adduct form with water or various other solvents, and these adducts are included in the present invention.

Optical isomers may exist with respect to Compound (I), and all the possible stereoisomers and their mixtures are also included in the scope of the present invention.

Specific examples of Compound (I) are shown in Table 1.

TABLE 1



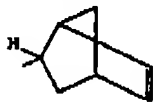
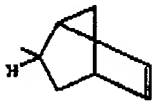
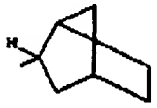
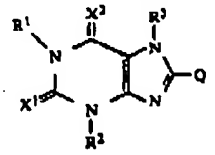
Compound No. (Example No.)	R^1	R^2	R^3	Q	X^1	X^2
1 (1)	n-C_3H_7	n-C_3H_7	H		O	O
2 (1)	n-C_3H_7	n-C_3H_7	H		O	O
3 (2)	n-C_3H_7	n-C_3H_7	H		O	O

TABLE 1-continued



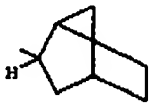
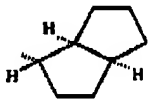
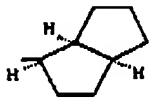
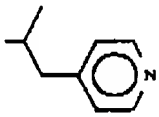
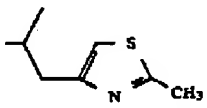
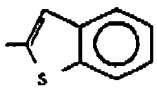
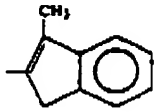
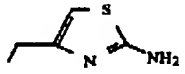
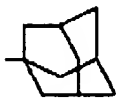
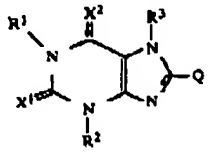
Compound No. (Example No.)	R^1	R^2	R^3	Q	X^1	X^2
4 (2)	n-C_3H_7	n-C_3H_7	H		O	O
5 (3)	n-C_3H_7	n-C_3H_7	H		O	O
6 (3)	n-C_3H_7	n-C_3H_7	H		O	O
7 (4)	n-C_3H_7	n-C_3H_7	H		O	O
8 (5)	n-C_3H_7	n-C_3H_7	H		O	O
9 (6)	n-C_3H_7	n-C_3H_7	H		O	O
10 (7)	n-C_3H_7	n-C_3H_7	H		O	O
11 (8)	n-C_3H_7	n-C_3H_7	H		O	O
12 (9)	n-C_3H_7	n-C_3H_7	H		O	O
13 (29)	n-C_3H_7	n-C_3H_7	H		O	O

TABLE 1-continued



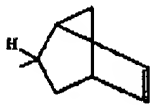
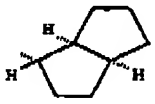
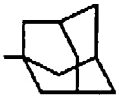
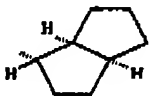
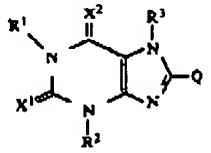
Compound No. (Example No.)	R^1	R^2	R^3	Q	X^1	X^2
14 (10)	n-C_3H_7	n-C_3H_7	H		O	O
15 (11)	n-C_3H_7	n-C_3H_7	H		O	O
16 (12)	n-C_3H_7	n-C_3H_7	H		O	O
17 (13)	n-C_3H_7	n-C_3H_7	CH_3		O	O
18 (14)	n-C_3H_7	n-C_3H_7	CH_3		O	O
19 (30)	n-C_3H_7	n-C_3H_7	CH_3		O	O
20 (15)	n-C_3H_7	n-C_3H_7	CH_3		O	O
21 (16)	n-C_3H_7	n-C_3H_7	C_2H_5		O	O
22 (17)	n-C_3H_7	n-C_3H_7	n-C_3H_7		O	O
23 (18)	H	n-C_3H_7	H		O	O

TABLE 1-continued



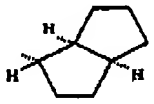
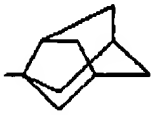
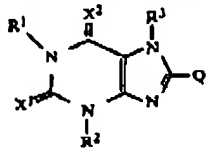
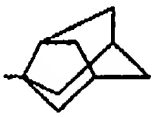
Compound No. (Example No.)	R^1	R^2	R^3	Q	X^1	X^2
24 (19)	n-C_3H_7	n-C_3H_7	H		S	O
25 (20)	H	n-C_3H_7	H		O	O
26 (21)	H	n-C_3H_7	H		O	S
27 (22)	CH_3	CH_3	H		O	O
28 (23)	C_2H_5	C_2H_5	H		O	O
29 (24)	n-C_4H_9	n-C_4H_9	H		O	O
30 (25)	CH_3	iso-C_4H_9	H		O	O
31 (26)	n-C_3H_7	n-C_3H_7	H		S	O
32 (27)	n-C_3H_7	n-C_3H_7	H		O	S
33 (28)	n-C_3H_7	n-C_3H_7	H		S	S
34 (31)	$CH_2{=}CHCH_2{-}$	$CH_2{=}CHCH_2{-}$	H		O	O

TABLE 1-continued



Compound No. (Example No.)	R^1	R^2	R^3	Q	X^1	X^2
35 (32)	$CH\equiv C-CH_2-$	$CH_2=CHCH_2-$	H		O	O

Compound (I) and its pharmaceutically acceptable salts have an activity of selectively antagonizing adenosine

A_1 receptor, and thus exhibit, a diuretic effect, a renal-protecting effect a bronchodilatory effect, etc. Compound (I) and its pharmaceutically acceptable salts are useful as a diuretic and renal-protecting agent, bronchodilatory agent, etc.

The pharmacological effects of Compound (I) are explained, referring to Test Examples.

Test Example 1, Acute Toxicity Test

A test compound (300 mg/kg) was orally administered to male dd-strain mice having a body weight of 20±1 g (3 animals/group). Minimum lethal dose (MLD) of the compounds was determined by observing whether or not the mice were alive after 7 days of the administration.

With respect to Compound Nos. 1—5, 7—11, 13—18, 20 23, 24 and 31—33, the MLD was more than 300 mg/kg, and with respect to Compound No. 12, that was 300 mg/kg. This shows the toxicity of Compound (I) is weak and can be administered safely over a wide range of dosage.

Test Example 2, Adenosine Receptor Binding Test

1) Adenosine A_1 Receptor Binding

This test was conducted according to the method of Bruns et al. [Proc. Natl. Acad. Sci., 77, 5547 (1980)] with some modification.

Cerebrum of a guinea pig was suspended into ice cooled 50 mM tris hydroxymethyl aminomethane hydrochloride (Tris HCl) buffer (pH=7.7), by using Polytron homogenizer (manufactured by Kinematica Co.). The suspension was centrifuged (50,000×g, 10 minutes), and the precipitate was resuspended by adding the same volume of 50 mM Tris HCl buffer. The suspension was centrifuged under the same conditions, and the precipitate obtained was suspended once again by adding 10 volumes of 50 mM Tris HCl The tissue suspension was incubated at 37° C. for 30 minutes in the presence of 0.02 units/mg tissue of adenosine deaminase (manufactured by Sigma Co.). The resulting tissue suspension was recentrifuged (50,000×g, 10 minutes), and 50 mM Tris HCl was added to the precipitate to adjust the concentration of tissue to 10 mg (wet weight)/ml.

To 1 ml of tissue suspension prepared above were added 50 μl of [^{3}H] cyclohexyladenosine [^{3}H-CHA, 27 Ci/mmol, manufactured by New England Nuclear Co.] (final concentration =1.1 nM) and 50 μl of test compound. The mixture was incubated at 25° C. for 90 minutes, and the resulting mixture was stopped by rapid vacuum filtration through a glass fiber filter (GF/C manufactured by Whatman Co.) and immediately washed three times with 5 ml each of ice cold 50 mM Tris HCl buffer. The filter was transferred to a vial bottle, and a scintillator (EX-H by Wako Pure Chemicals Industries, Ltd.) was added thereto. Its radioactivity was then determined by a scintillation counter (manufactured by Packard Instrument Co.).

The inhibition rate of the test compound against the binding of A1 acceptor (^{3}H-CHA binding) was calculated from the following equation:

$$\text{Inhibition (\%)} = \left(1 - \frac{[B]-[N]}{[T]-[N]}\right) \times 100$$

[Notes]
1. "B" means the radioactivity of ^{3}H-CHA bound in the presence of a test compound at a concentration shown in Table 2.
2. "T" means the radioactivity of ^{3}H-CHA bound in the absence of test compounds.
3. "N" means the radioactivity of ^{3}H-CHA bound in the presence of 10 μM of N^6-(L-2-phenylisopropyl)adenosine (manufactured by Sigma Co.).

The results are shown in Table 2. The inhibition constant (Ki value) shown in the table was calculated from Cheng-Prusoff's equation. 2) Adenosine A_2 Acceptor Binding Test

This test was conducted according to the method of Bruns et al. [Mol. Pharmacol., 29, 331 (1986)] with some modification.

A precipitate was prepared from rat corpus striatum in a similar manner as in 1) above. The precipitate was suspended by adding a 50 mM Tris HCl buffer containing 10 mM magnesium chloride and 0.02 unit/mg (tissue) of adenosine deaminase (manufactured by Sigma Co.) to adjust the concentration of tissue to 5 mg (wet weight)/ml.

To 1 ml of tissue suspension prepared above were added 50 μl of a mixture of N-ethylcarboxamidoadenosine [^{3}H-NECA, 26 Ci/mmol, manufactured by Amersham Co.] (final concentration=3.8 nM) and cyclopentyladenosine [CPA, manufactured by Sigma Co.] (final concentration=50 nM), and 50 μl of test compound. The mixture was incubated at 25° C. for 120 minutes. The resulting mixture was treated in the same manner as in 1) above to determine its radioactivity.

The inhibition rate of the test compound against the binding of A2 receptor (^{3}H-NECA binding) was calculated from the following equation:

$$\text{Inhibition Rate (\%)} = \left(1 - \frac{[B]-[N]}{[T]-[N]}\right) \times 100$$

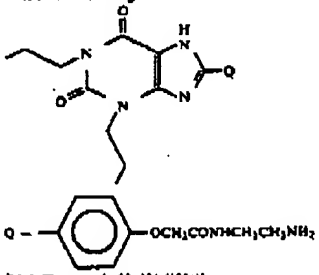
-continued

[Notes]
IC_{50}: Concentration at an inhibition rate of 50%
L: Concentration of ^{3}H-NECA
Kd: Dissociation constant of ^{3}H-NECA
C: Concentration of CPA.
Kc: Inhibition constant of CPA

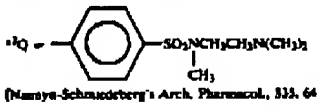
TABLE 2

Compound No.	A_1 Receptor: Inhibition (%)/ Concentration of Tested Compound [10^{-3}/10^{-4}M]	A_1 Receptor: Ki (nM)	A_2 Receptor: Inhibition (%)/ Concentration of Tested Compound [10^{-3}/10^{-4}M]	A_2 Receptor: Ki (nM)	Ratio of Ki Values [A_2/A_1]
1	99/99	5.5	88/97	510	92.7
3	100/100	4.4	83/90	330	75.0
5	99/99	3.8	91/99	330	86.8
6	100/101	5.0	70/85	560	112
13	100/100	7.8	63/71	1,400	179
14	101/101	1.3	63/77	380	292
28	100/101	7.1	61/78	940	132
29	100/100	9.1	72/78	970	107
XAC*1	98(10^{-6}M)	11	99/—	21	1.91
PD 115199*2	97/100	190	94/98	26	0.14
CGS 15943*3	99/96	10	99/97	0.73	0.073
Theophylline	33/74	13,000	26/69	20,000	0.87

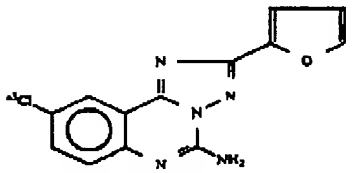
[Notes]
*1Xanthine derivative



[Mol. Pharmacol., 29, 126 (1986)]



[Naunyn-Schmiedeberg's Arch. Pharmacol., 333, 64 (1987)]



[J. Med. Chem., 31, 1014 (1988)]

[Notes]
1. "B" means the radioactivity of ^{3}H-NECA bound in the presence of a test compound at a concentration shown in Table 2.
2. "T" means the radioactivity of ^{3}H-NECA bound in the absence of test compounds.
3. "N" means the radioactivity of ^{3}H-NECA bound in the presence of 100 μM of CPA.

The results are shown in Table 2. The Ki values shown in the table were calculated from the following equation:

$$Ki = \frac{IC_{50}}{1 + \frac{L}{Kd} + \frac{C}{Kc}}$$

Test Example 3, Diuretic Effect

Wistar rats (male: 150–300 g) were starved for 18 hours prior to the administration of the test compound. A test compound (25 mg/kg) and saline (25 ml/kg) were orally administered to test rats and only saline was administered to control rats. Three groups, each group consisting of 3 rats, were used for each test compound. Urine was collected for 6 hours after the administration. Urine volume was measured and the electrolytes (Na^+ and K^+) in the urine wee determined with a flame photometer (775A, Hitachi Ltd., Japan). The results are shown in Table 3.

All parameters are expressed as relative values of control.

TABLE 3

Compound No.	Increase in Urine (%)	Increase in Na^+ excretion (%)	Increase in K^+ excretion (%)	Na^+/K^+
(Control)	0	0	0	1.00
1	106	73	36	1.27
2	87	109	67	1.25
3	134	137	29	1.84
4	113	106	27	1.63
5	88	109	32	1.58
6[a3]	330	252	87	1.88
8	82	138	22	1.95
12	109	103	32	1.54
13	129	186	37	2.09
14	315	244	68	2.03
16	141	191	38	2.12
17	155	107	51	1.37
24	112	128	61	1.40
29	123	137	65	1.43
30	112	126	50	1.50
31	115	126	36	1.44
32	100	114	41	1.51
33	99	105	40	1.47
Aminophylline[1] (Reference compound)	34	89	17	1.62
Furosemide[2] (Reference compound)	73	64	57	1.07

[1]The Merck Index. 10th edition, page 476 (1983)
[2]The Merck Index. 10th edition, page 4189 (1983)
[3]The amount of the administration: 6.25 mg/kg

Test Example 4, Renal-Protecting Effect (Glycerol-Induced Renal Failure Model)

A renal failure is a state where the renal function is lowered and the homeostasis of a body fluid can be no more maintained. It is known that an acute renal failure characteristic of uriniferous tubule disorder is caused by subcutaneous or intramuscular injection of glycerol to rats [Can. J. Physiol. Pharmacol., 65, 42 (1987)].

Male Wistar rats were kept deprived of water for 18 hours, and served for the test. A test compound was intraperitoneally administered to the rats (dosage: 1 ml/kg) and the rats were anesthetized with ether and 50% glycerol was subcutaneously administered (dosage: 0.8 ml/100 g) to the rats, pinching the dorsal skin. Twenty four hours after the administration of glycerol, the rats were anesthetized with ether and 5 ml of blood was collected from the abdominal aorta. The collected blood was allowed to stand for 30 minutes or longer and then centrifuged at 3,000 rpm for 10 minutes, and the amounts of the serum creatinine and urine-nitrogen (UN) contained in a serum were determined by auto analyzer (Olympus AU510) or measured by the creatinine test Wako (Jaffe method) and UN Test Wako (diacetylmonooxime direct method). Both are manufactured by Wako Pure Chemicals Co.

On the other hand, the left kidneys of the blood-sampled rats were removed and placed in formalin-filled vial bottles, and used as samples for the pathological examination.

According to the test results, Compound Nos. 1—5, 7, 8, 13, 14, 16, 17, 23, 25 and 31 significantly suppressed increases in the serum creatinine and in urine-nitrogen, when administered abdominally at a dosage of 0.01 - 10 mg/kg [i.p.] ($p < 0.05$) whereas XAC and aminophylline had a weak effect of suppressing the increase, and PD 115,199 and CGS15,943 were totally invalid. On the contrary, furosemide showed a tendency to increase the serum creatinine. The pathological examination of removed kidneys indicates that compounds Nos. 1 —5, 7, 8, 13, 14, 16, 17, 23, 25 and 31 also significantly improved the state of kidneys.

Test Example 5, Effects On Passive Schultz-Dale Reaction

Male Hartley guinea pigs weighing 350 to 500 g were passively sensitized by intraperitoneal injection of rabbit anti-egg white albumin (EWA) serum prepared by the method of Koda, et al. [Folia pharmacol. Japan 66, 237 (1970)]. After 24 h the guinea pigs were stunned and exsanguinated, and then the trachea were removed. The zig-zag strips of the trachea were prepared by the method of Emmerson, et al. [J. Pharm. Pharmacol., 31, 798 (1979)]. The strips were suspended in Krebs-Henseleit solution at 37° C. aerated with 95% O_2 and 5% CO_2, and incubated for one hour. Antigen (EWA) was then introduced in the solution (final concentration; 1 μg/ml), and the contraction was measured by isotonic-transducer (TD-112s, Nihon Kohden, Japan) and recorded on a recorder (Type 3066, Yokogawa-Hokushin Denki, Japan). After the contraction reached a stable plateau, the compounds were cumulatively added in order to get concentration-relaxation curves. Concentration of compounds to produce 50% relaxation (IC_{50}) was calculated from the regression curve, obtained from cumulative concentration-relaxation response. The results are shown in Table 4.

TABLE 4

Compound No.	Passive 5-D Reaction IC_{50} (μM)	MED (mg/kg) for Inhibiting Death Induced by PAF
7	2.7	>100
8	0.88	100
23	17.3	—
26	22.7	—
Theophylline	23	100

Test Example 6, Effect of Inhibiting Death Induced By Platelet-Activating Factor (PAF)

A test compound (100 mg/kg) was orally administered to dd strain mice (male animals, 28 to 32 g) and 40 μg/kg of PAF (manufactured by Avanti Polar Lipids Co.) was administered via tail veins 1 hour after the administration according to the method of Carlson et al. [Agents and Actions, 21, 379 (1987)]. The mortality rates of compound-treated groups were compared with those of matched control groups, assessed during the same experimental session, by the Fisher's exact probability test. The cases wherein the level of significance (p value) is 0.05 or less are considered to be effective with respect to the inhibition. The above procedure was repeated, using the test compound in a decreasingly small quantity so as to find out the Minimum Effective Dosage (MED) wherein no significant difference be observed between the test and control groups.

The results are shown in Table 4.

Test Example 7, Acute Toxicity Test

A test compound was orally administered to male dd-strain mice having a body weight of 20±1 g (3 animals/group). Minimum lethal dose (MLD) of the compound was determined by observing whether or not the mice were alive after 7 days of the administration.

With respect to Compound 34, the MLD was more than 300 mg/kg. This shows the toxicity of Compound

(I) is weak and can be administered safely over a wide range of dosage.

Test Example 8, Adenosine A_1 Receptor Binding Test

This test was conducted according to the method of Bruns et al. [Proc. Natl. Acad. Sci., 77, 5547 (1980)] with some modification.

Cerebrum of a guinea pig was suspended into ice cooled 50 mM tris hydroxymethyl aminomethane hydrochloride (Tris HCl) buffer (PH 7.7), by using Polytron homogenizer (manufactured by Kinematica Co.). The suspension was centrifuged (50,000×g, 10 minutes), and the precipitate was resuspended by adding the same volume of 50 mM Tris HCl buffer. The suspension was centrifuged under the same conditions, and the precipitate obtained was suspended once again by adding 50 mM Tris HCl until the concentration of tissue was 100 mg (wet weight)/ml. The tissue suspension was incubated at 37° C. for 30 minutes in the presence of 0.02 units/mg tissue of adenosine deaminase (manufactured by Sigma Co.). The resulting tissue suspension was recentrifuged (50,000×g, 10 minutes), and 50 mM Tris HCl was added to the precipitate until the concentration of tissue was 10 mg (wet weight)/ml.

To 1 ml of tissue suspension prepared above were added 50 µl of [^{3}H] cyclohexyladenosine [^{3}H-CHA, 27 Ci/mmol, manufactured by New England Nuclear Co.] (final concentration: 1.1 nM) and 50 µl of the test compound. The mixture was incubated at 25° C. for 90 minutes, and the resulting mixture was stopped by rapid vacuum filtration through a glass fiber filter (GF/C manufactured by Whatman Co.) and immediately washed three times with 5 ml each of ice cold 50 mM Tris HCl buffer. The filter was transferred to a vial bottle, and a scintillator (EX-H by Wako Pure Chemical Industries, Ltd.) was added thereto. Its radioactivity was then determined by a scintillation counter (manufactured by Packard Instrument Co.).

The inhibition rate of the test compound against the binding of A_1 receptor (^{3}H-CHA binding) was calculated from the following equation:

$$\text{Inhibition (\%)} = (1 - ((B) - (N))/((T) - (N))) \times 100$$

[Notes]

1. "B" means the radioactivity of ^{3}H-CHA bound in the presence of a test compound at a concentration shown in Table 1.

2. "T" means the radioactivity of ^{3}H-CHA bound in the absence of a test compound.

3. "N" means the radioactivity of ^{3}H-CHA bound in the presence of 10 µM of N^6-(L-2-phenylisopropyl-)adenosine (manufactured by Sigma Co.).

The results are shown in Table 5. The inhibition constant (Ki value) shown in the table was calculated from Cheng-Prusoff's equation.

TABLE 5

	A_1 Receptor	
Compound No.	Inhibition (%)/ Concentration of Test Compound [10^{-5}/10^{-4}M]	Ki (nM)
34	99/99	15

According to the result, Compound 34 almost perfectly inhibited against ^{3}H-CHA binding (the binding of A_1 receptor).

Test Example 9, Diuretic Effect

Wistar rats (male: 150-300 g) were starved for 18 hours prior to the administration of a test compound. The test compound was suspended in saline, and the test compound was orally administered to rats at a concentration of 0.1 to 10 mg/25 ml/kg. Only saline was administered to rats. The rats to which only saline was administered was made as control group. Three groups, each group consisting of 3 rats, were used for each test. Urine was collected for 6 hours after the administration. Urine volume was measured and the electrolytes (Na^+ and K^+) in the urine were determined with a flame photometer (775A, Hitachi Ltd., Japan). The results are shown in Table 6.

All parameters are expressed as relative values of control.

TABLE 6

Compound No.	The amount of the administration (mg/kg)	Increase in Urine (%)	Increase in Na^+ excretion (%)	Increase in K^+ excretion (%)	Na^+/K^+
(Control)	—	0	0	0	1.00
34	6.25	82	71	18	1.45
34	0.40	109	99	8	1.84
Aminophylline[1] (Reference compound)	25	34	89	17	1.62
Furosemide[2] (Reference compound)	25	75	64	57	1.07

[1]The Merck Index, 11th edition, page 76 (1989)
[2]The Merck Index, 11th edition, page 674 (1989)

The result indicates that diuretic effect of Compound 34 is higher than that of Aminophylline or Furosemide.

Test Sample 10 Renal-Protecting Effect (Glycerol-Induced Renal Failure Model)

A renal failure is a state where the renal function is lowered and the homeostasis of a body fluid can no longer be maintained. It is known that an acute renal failure characteristic of uriniferous tubule disorder is caused by subcutaneous or intramuscular injection of glycerol to rats [Can. J. physiol. pharmacol., 65, 42 (1987)].

Male Wistar rats were kept deprived of water for 18 hours, and served for the test. A test compound was intraperitoneally administered to the rats (dosage: 0.1 mg/1 ml/(saline)/kg) and the rats were anesthetized with ether and 50% glycerol was subcutaneously administered (dosage: 0.8 mg/100 g) to the rats, pinching the dorsal skin. Twenty four hours after the administration of glycerol, the rats were anesthetized with ether and 5 ml of blood was collected from the abdominal aorta. The collected blood was allowed to stand for 30 minutes or longer and then centrifuged at 3,000 rpm for 10 minutes, and the amounts of the serum creatinine and the serum urine-nitrogen (UN) were determined by auto analyzer (Olympus AU510) [creatine test (Jaffe method), UN test (enzyme method); both tests were used in Olympus AU500/550 exclusive reagent] or mea-

sured by the Creatinine Test Wako (Jaffe method) and UN Test Wako (diacetylmonooxime direct method). Both are manufactured by Wako Pure Chemicals Co.

On the other hand, the left kidneys of the blood-sampled rats were removed and placed in formalin-filled vial bottles, and pathologically examined in contrast with the left kidneys of test compound-untreated rats.

The results are shown in following Table 7.

TABLE 7

Compound No.	The amount of the administration (mg/kg)	The amount of the serum creatinine (mg/dl) Control group	Test compound administered-group	The amount of the serum urine-nitrogen (mg/dl) Control group	Test compound administered-group
34	0.1	4.94 ± 0.05	2.15 ± 0.22(***)	174.8 ± 4.0	78.6 ± 9.7(***)
Aminophylline (Reference compound)	10	2.03 ± 0.18	1.72 ± 0.07	46.2 ± 6.5	30.6 ± 2.0(**)
Furosemide (Reference compound)	10	3.22 ± 0.35	4.17 ± 0.41	110.7 ± 9.4	150.3 ± 13.3(**)
Control	—	0.50 ± 0.02	—	15.2 ± 0.9	—

(***P < 0.001, **P < 0.05)

Values in test compound-treated group were compared to those in control group using the Student's t-test (n = 8 — 10).

According to the results, Compound 34 significantly suppressed increases in the serum creatinine and in serum urine-nitrogen, when administered abdominally at a dosage of 0.1 mg/kg [i.p.] whereas aminophylline (10 mg/kg) had a weak effect of suppressing the increase. On the contrary, furosemide (10 mg/kg) showed a tendency to increase the serum creatinine. The pathological examination of removed kidney indicates that compound 34 also significantly improved the state of kidney.

Compound (I) or its pharmaceutically acceptable salts can be used as such or in various medicament forms. The present pharmaceutical composition can be prepared by uniformly mixing an effective amount of Compound (I) or its pharmaceutically acceptable salts as an active component with a pharmaceutically acceptable carrier. The pharmaceutical composition is desirably in a unit dosage form applicable to oral or injection administration.

In the preparation of pharmaceutical compositions in an oral dosage form, some useful, pharmaceutically acceptable carrier can be used. For example, liquid, orally administerable compositions such as suspension compositions or syrup compositions can be prepared with water, a saccharide such as sucrose, sorbitol, fructose, etc., a glycol such as polyethyleneglycol, propyleneglycol, etc., an oil such as sesame oil, olive oil, soybean oil, etc., an antiseptic such as p-hydroxybenzoic acid esters, etc., and a flavor such as strawberry flavor, peppermint, etc. Powder, pills, capsules and tablets can be prepared with a vehicle such as lactose, glucose, sucrose, mannitol, etc., a disintegrator such as starch, sodium alginate, etc., a lubricant such as magnesium stearate, talc, etc., a binder such as polyvinyl alcohol, hydroxypropyl cellulose, gelatin, etc., a surfactant such as fatty acid esters, etc., a plasticizer such as glycerin, etc. and so forth. Tablets and capsules are most useful unit for oral administration because of easy administration. In the preparation of tablets or capsules, a solid pharmaceutical carrier is used.

Injection solutions can be prepared with a carrier such as distilled water, saline solution, glucose solution, or a mixture of saline solution and glucose solution.

Effective dosage and number of administration of Compound (I) or its pharmaceutically acceptable salts depend on the administration route and ages, body weights, symptoms, etc. of patients, and it is preferable to usually administer Compound (I) at a dosage of 1 to 50 mg/kg per day in 3 to 4 divisions.

Compound (I) and pharmacologically acceptable salts thereof can also be administered by inhalation in the form of aerosol, finely divided powders or sprayed mist. In the case of aerosol administration, the compounds according to the invention can be dissolved in an appropriate, pharmacologically acceptable solvent (e.g., ethyl alcohol) or a mixture of miscible solvents, and then admixed with a pharmacologically acceptable propellant. Such an aerosol composition can be charged in a pressure container equipped with an appropriate aerosol valve suited for the spraying of the aerosol composition. It can be preferable to use an aerosol valve which is capable of spraying a predetermined quantity of aerosol composition to provide an effective dosage thereof.

The present invention will be described below, by the following Examples and Reference Examples.

EXAMPLE 1

8-[(1R*,4S*,5S*)-2-Bicyclo[2.2.1]hepten-5-yl]-1,3-dipropylxanthine (Compound 1), and 8-[(1R*, 4S*, 5R*)2-bicyclo[2.2.1]hepten-5-yl]-1,3-dipropylxanthine (Compound 2)

At first, 2.57 g (18.6 mmol) of bicyclo[2.2.1]-5-heptane-2-carboxylic acid and 3.06 g (16.0 mm (3-dimethylaminopropyl)carbodiimide hydrochloride were added to a solution of 3.00g (13.3 mmol) of 1,3-dipropyl-5,6-diaminouracil [U.S. Pat. No. 2,607,295 and J. Org. Chem., 16, 1879 (1951)] in 60 ml of dioxane and 30 ml of water and the mixture was stirred at room temperature for 1 hour, while adjusting the pH to 5.5. The pH of the mixture is adjusted to 7.0, and the mixture was extracted with chloroform three times, and the extract was washed with a saturated aqueous sodium chloride and dried over anhydrous sodium sulfate. Then, the solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography (eluent: 2.0% methanol/chloroform) to afford 4.07 g (yield: 88%) of amorphous 6-amino-5-(2-bicyclo[2,2,1]hepten-5-yl)carbonylamino-1,3-dipropyluracil.

NMR ($CDCl_3$, 90 MHz) δ(ppm): 7.20 (brs, 1H), 6.22–5.95 (m, 2H), 5.35 (brs, 2H), 4.00–3.65 (m, 4H), 3.52–2.80 (m, 3H) and 2.20–0.80 (m, 14H)

Then, 40 ml of dioxane and 40 ml of 2N sodium hydroxide aqueous solution were added to 3.99 g (11.5 mmol) of the thus obtained compound and the mixture was refluxed under heating for 20 minutes. After cooling, the mixture was neutralized and extracted with chloroform three times. Then, the extract was washed with a saturated aqueous sodium chloride and dried over anhydrous sodium sulfate, and then the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: 25% ethyl acetate/hexane) and recrystallized from cyclohexane to afford 1.97 g (yield: 52%) of the captioned Compound 1 as a white powder and 0.63 g (yield: 18%) of the captioned Compound 2 as a white powder.

Compound 1

Melting point: 121.6°–122.8° C. (recrystallized from isopropanol/water)

Rf value: 0.30 [TLC plate silica gel $60F_{254}$ (product of Merck Co., eluent: 30% ethylacetate/hexane]

Elemental analysis: $C_{18}H_{24}N_4O_2$

Calculated (%): C 65.83, H 7.37, N 17.06

Found (%) C 65.71, H 7.31, N 16, 78

IR (KBr) νmax (cm^{-1}): 1,698, 1,653, 1,497

NMR (DMSO-d_6) δ(ppm): 12.84 (s, 1H), 6.17 (dd, J=3.2, 5.6 Hz, 1H), 5.72 (dd, J=2.7, 5.6 Hz, 1H), 3.91 (t, 2H), 3.82 (t, 2H), 3.43 (ddd, J=4.2, 4.2, 9.3 Hz, 1H), 3.28 (brs, 1H), 2.92 (brs, 1H), 2.08 (ddd, J=3.7, 9.3, 13.0 Hz, 1H), 1.75–1.50 (m, 5H), 1.45–1.35 (m, 2H) and 0.90–0.80 (m, 6H)

Compound 2

Melting point: 167.6°–168.0° C. (recrystallized from ethanol/water)

Elemental analysis: $C_{18}H_{24}N_4O_2$

Calculated (%): C 65.83, H 7.37, N 17.06

Found (%) C 66.03, H 7.69, N 17.09

Rf value: 0.46 (30% ethyl acetate/hexane)

IR (KBr) νmax (cm^{-1}): 1,695, 1,657, 1,495

NMR (DMSO-d_6) δ(ppm): 13.11 (brs, 1H), 6.21 (d, J=1.4 Hz, 2H), 3.95 (t, 2H), 3.84 (t, 2H), 2.96 (brs, 2H), 2.63 (ddd, J=0.7, 4.2, 8.2 Hz, 1H), 2.10 (ddd, J=4.2, 4.2, 11.5 Hz, 1H), 1.75–1.45 (m, 5H), 1.35–1.22 (m, 2H), 0.92–0.80 (m, 6H).

EXAMPLE 2

8-[(1R*,2S*,5S*)-Bicyclo[2.2.1]heptan-2-yl]-1,3-dipropylxanthine (Compound 3) and 8-[(1R*, 2R*, 5S*)-bicyclo[2.2.1]heptan-2-yl]-1,3-dipropylxanthine (Compound 4)

The substantially same operations as in Example 1 were repeated using 3.0g (13.3 mmol) of 1,3-dipropyl-5,6-diaminouracil and 2.61g (18.6 mmol) of bicyclo[2.2.1]heptane-2-carboxylic acid to afford 4.31g (yield: 93%) of amorphous 6-amino-5-(bicyclo[2.2.1-]heptan-2-yl)carbonylamino-1,3-dipropyluracil.

NMR ($CDCl_3$, 90 MHz) δ(ppm): 7.21 (brs, 1H), 5.40 (brs, 25, 2H), 4.00–3.70 (m, 4H), 3.00 –2.75 (m, 1H), and 2.65–0.75 (m, 20H)

The substantially same cyclization reaction as in Example 1 was performed using 4.30g (12.3 mmol) of the thus obtained compound, to afford 3.05g (yield: 75%) of 8-bicyclo[2.2.1]heptan-2-yl)-1,3-dipropylxanthine [a mixture of (1R*, 2S*, 5S*) isomer (Compound 3) and (1R*, 2R*, 5S*) isomer (Compound 4)] as a white powder. The mixture was subjected to high performance liquid chromatography (HPLC) [column, R-354 (30 cm×50 mmφ) (by Yamamura Kagaku K. K.); eluent, 85% methanol/water; flow rate, 50 ml/min.] to afford 327 mg of the captioned Compound 3 and 442 mg of the captioned Compound 4.

Compound 3

Melting point: 150.9°–152.0° C. (recrystallized from isopropanol/water)

Elementary analysis: $C_{18}H_{26}N_4O_2$

Calculated (%): C 65.43, H 7.93, N 16.96

Found (%) C 65.41, H 8.11, N 17.00

IR (KBr) νmax (cm^{-1}): 1700, 1650, 1497.

NMR (DMSO-d6) δ(ppm): 13.00 (brs, 1H), 3.97 (t, 2H),

3.84 (t, 2H), 3.21 (ddd, J=4.2, 4.2, 11.6 Hz, 1H), 2.55 (brs, 1H), 2.28 (brs, 1H), 1.90–1.22 (m, 11H), 1.15–1.03 (m, 1H), 0.95–0.82 (m, 6H).

HPLC [AM-312 (15 cm×5 mmφ) (by Yamamura Kagaku K. K.),

70% acetonitrile-water, UV 254 nm, 1.0 ml/min]:

Retention time; 12.7 min.

Compound 4

Melting point: 139.7°–142.9° C. (recrystallized from isopropanol/water)

Elementary analysis: $C_{18}H_{26}N_4O_2$

Calculated (%): C 65.43, H 7.93, N 16.96

Found (%) C 65.66, H 8.29, N 16.90

IR (KBr) νmax (cm^{-1}): 1702, 1650, 1494.

NMR (DMSO-d_6) δ(ppm): 12.99 (brs, 1H), 3.94 (t, 2H), 3.83 (t, 2H), 2.79 (dd, J=4.9,8.5 Hz, 1H), 2.39 (brs, 1H), 2.31 (brs, 1H), 2.08–1.96 (m, 1H), 1.80–1.45 (m, 8H), 1.38–1.12 (m, 3H), 0.95–0.80 (m, 6H).

HPLC [AM-312 (15 cm×5 mmφ) (by Yamamura Kagaku K. K.)

70% acetonitrile-water, UV 254 nm, 1.0 ml/min]:

Retention time; 13.9 min.

In Examples 3 to 9 described below, the desired compounds were obtained in the substantially same operations as in Example 1, except that a corresponding carboxylic acid was used instead of bicyclo[2.1.1]-5-heptene-2-carboxylic acid. In those examples, intermediates obtained were used in the subsequent cyclization reactions without being isolated or purified.

EXAMPLE 3

8-[(1R*,2R*,5R*)-Bicyclo[3.3.0]octan-2-yl]-1,3-dipropylxanthine (Compound 5) and bicyclo[3.3.0]oxtan-2-yl]-1,3-dipropylxanthine (Compound 6)

The substantially same operations as in Example 1 were repeated using 4.55 ml (31.9 mmol) of bicyclo[3.3.-0]octane-2-carboxylic acid, and the following two compounds were obtained.

Compound 5

Yield: 4.30 g (Yield, 47%; white plate crystal)

Melting point: 100.1°–101.6° C. (recrystallized from heptane)

Rf value: 0.53 (30% ethyl acetate/hexane)

Elementary analysis: $C_{19}H_{28}N_4O_2$

Calculated (%): C 66.25, H 8.19, N 16.27

Found (%) C 66.07, H 8.43, N 16.61

IR (KBr) νmax (cm^{-1}): 1,699, 1,653 and 1,499
NMR(DMSO-d6) δ(ppm): 13.12 (brs, 1H), 3.94 (t, 2H), 3.83 (t, 2H), 2.75–2.50 (m, 3H), 2.10–1.45 (m, 12H), 1.42–1.35 (m, 1H), 1.30–1.15 (m, 1H), 0.95–0.85 (m, 6H)
^{13}C-NMR ($CDCl_3$) δ(ppm): 159.1, 155.7, 151.1, 149.4, 106.7, 50.4, 47.6, 45.3, 43.4, 43.2, 34.4, 34.1, 33.6, 32.1, 25.1, 21.4, 11.4, 11.2.

Compound 6

Yield: 359 mg (Yield, 3.9%; white plate crystal)
Melting point: 118.4°–120.0° C. (recrystallized from heptane)
Rf value: 0.40 (30% ethyl acetate/hexane)
Elementary analysis: $C_{19}H_{28}N_4O_2$
Calculated (%): C 66.25, H 8.19, N 16.27
Found (%) C 66.20, H 8.63, N 16.31
IR (KBr) νmax (cm^{-1}): 1,699, 1,652 and 1,497
NMR ($CDCl_3$) δ(ppm): 12.30 (brs, 1H), 4.11 (t, 2H), 4.02 (t, 2H), 3.30 (ddd, 1H, J=6.8, 14 Hz), 3.00–2.85 (m, 1H), 2.70–2.53 (m, 1H), 2.25–0.90 (m, 20H) ^{13}C-NMR ($CDCl_3$) δ(ppm): 157.0, 155.5, 151.2, 149.2, 106.5, 47.6, 45.2, 44.0, 43.3, 42.9, 35.4, 32.5, 29.7, 27.5, 27.4, 21.4, 21.4, 11.4, 11.2.
MS (m/e) relative intensity: 344 (M+, 100), 302 (28), 260 (18), 250 (23) and 230 (18)

EXAMPLE 4

8-[1-methyl-2-(4-pyridyl)ethyl]-1,3-dipropylxanthine

(Compound 7)

Overall yield: 79% (White needle crystal)
Melting point: 214.9°–217.3° C.
Elementary analysis: $C_{19}H_{25}N_5O_2.HCl.0.1H_2O$
Calculated (%): C 57.74, H 6.64, N 17.72
Found (%) C 57.79, H 6.54, N 17.63
IR (KBr) νmax (cm^{-1}) 1,704, 1,669 and 1,637
NMR (DMSO-d_6) δ(ppm): 13.50–12.80 (brs, 1H), 8.79 (d, 2H, J=6.1 Hz), 7.84 (d, 2H, J=6.1 Hz), 3.90 (t, 2H), 3.81 (t, 2H), 3.50–3.20 (m, 3H), 1.70–1.50 (m, 4H), 1.33 (d, 3H, J=6.7 Hz), 0.85 (t, 3H), 0.82 (t, 3H)

EXAMPLE 5

8-[1-Methyl-2-(2-methylthiazol-4-yl)ethyl]-1,3-dipropylxanthine

(Compound 8)

Overall yield: 70% (White plate crystal)
Melting point: 137.6°–139.2° C. (recrystallized from cyclohexane)
Elementary analysis: $C_{18}H_{25}N_5O_2S$
Calculated (%): C 57.58, H 6.71, N 18.65
Found (%) C 57.75 H 6.72, N 18.48
IR (KBr) νmax (cm^{-1}): 1,698, 1,659 and 1,499
NMR (DMSO-d_6) δ(ppm): 13.11 (brs, 1H), 7.00 (s, 1H), 3.94 (t, 2H), 3.83 (t, 2H), 3.45–3.10 (m, 3H), 2.92 (dd, 1H, J=6.8, 14.2 Hz), 2.59 (s, 3H), 1.75–1.50 (m, 4H), 1.23 (d, 3H, J=6.8 Hz), 0.95–0.80 (m, 6H)

EXAMPLE 6

8-(Benzo[b]thiophen-2-yl)-1,3-dipropylxanthine

(Compound 9)

Overall yield: 61% (White needle crystal)
Melting point: 307.9°–309.1° C. (recrystallized from ethanol)
Elementary analysis: $C_{19}H_{20}N_4O_2S$
Calculated (%): C 61.94, H 5.47, N 15.21
Found (%) C 61.91, H 5.44, N 15.15
IR (KBr) νmax (cm^{-1}): 1,699, 1,642 and 1,537
NMR (DMSO-d_6) δ(ppm): 8.19 (s, 1H), 8.05–7.85 (m, 2H), 7.50–7.40 (m, 2H), 4.00 (t, 2H), 3.87 (t, 2H), 1.85–1.50 (m, 4H), 1.00–0.80 (m, 6H) 8-(Benzo[b]furan-2-yl)-1,3-dipropylxanthine

(Compound 10)

Overall yield: 71% (White needle crystal)
Melting point: 282.1°–283.9° C. (recrystallized from ethanol)
Elementary analysis: $C_{19}H_{20}N_4O_3$
Calculated (%): C 64.76, H 5.72, N 15.90
Found (%): C 64.80, H 5.72, N 15.77
NMR (DMSO-d_6) δ(ppm): 14.30 (brs, 1H), 7.80–7.65 (m, 2H), 7.68 (s, 1H), 7.50–7.30 (m, 2H), 4.02 (t, 2H), 3.88 (t, 2H), 1.85–1.50 (m, 4H), 1.00–0.80 (m, 6H)

EXAMPLE 8

8-(3-Methylinden-2-yl)-1,3-dipropylxanthine

Compound 11)

Overall yield: 36% (Light yellow plate crystal)
Melting point: 268.1°–269.9° C. (recrystallized from ethanol)
Elementary analysis: $C_{21}H_{24}N_4O_2$
Calculated (%): C 69.21, H 6.64, N 15.37
Found(%) C 69.40, H 6.72, N 15.34
IR (KBr) νmax (cm^{-1}): 1,690, 1,641 and 1,485
NMR (DMSO-d_6) δ(ppm): 13.33 (brs, 1H), 7.55–7.45 (m, 2H), 7.40–7.25 (m, 2H), 4.04 (t, 2H), 3.88 (s, 2H), 3.90–3.80 (m, 2H), 2.61 (s, 3H), 1.85–1.50 (m, 4H), 0.95–0.80 (m, 6H)

Example 9

8-(2-Aminothiazol-4-yl)methyl-1,3-dipropylxanthine

(Compound 12

Overall yield: 94% (Light yellow plate crystal)
Melting point: 282.5°–284.3° C. (recrystallized from isopropanol)
IR (KBr) νmax (cm^{-1}) 1,697, 1,660, 1,523 and 1,500
NMR (DMSO-d6) δ (ppm): 13.28 (brs, 1H), 6.89 (brs, 2H), 6.23 (s, 1H), 4.00–3.80 (m, 4H), 3.86 (s, 2H), 1.80–1.50 (m, 4H), 0.95–0.80 (m, 6H)
MS (m/e) (Relative intensity): 348 (M+, 100), 306 (51), 277 (26), 264 (47), 248 (28), 234 (86) and 113 (38)

Example 10

8-(Noradamantan-3-yl)-1,3-dipropylxanthine

(Compound 14)

At first, 1.62 g (9.74 mmol) of 3-noradamantanecarboxylic acid was dissolved in 30 ml of pyridine, and 0.78 ml (10.7 mmol) of thionyl chloride was gradually added thereto at 0° C. After the mixture was stirred for 30 minutes at room temperature, 2.00 g (8.85 mmol) of 1,3-dipropyl-5,6-diaminouracil was gradually added thereto at 0° C. After stirring for 30 minutes at 0° C., the mixture was treated in the same procedure as in Example 29, and the residue was subjected to silica gel column chromatography (eluent: 1% methanol/-chloroform) to afford 3.55 g (yield: 100%) of amorphous 6-amino-5-(noradamantane-3-carbonylamino)-1,3-dipropyluracil.

NMR (90 MHz; $CDCl_3$) δ(ppm): 7.38 (brs, 1H), 5.62 (brs, 2H), 4.00–3.70 (m, 4H), 2.90–2.60 (m, 1H), 2.40 –1.30 (m, 16H), 1.10–0.80 (m, 6H)

The substantially same cyclization reaction as in Example 29 was performed by reacting 2.90 g (7.55 mmol) of the thus obtained compound with phosphorus oxychloride to afford 509 mg (yield: 14%) of the captioned Compound 14 as a white needle crystal.

Melting point: 190.0°–191.0° C. (recrystallized from heptane)

Elementary analysis: $C_{20}H_{28}N_4O_2$

Calculated (%): C 67.39, H 7.92, N 15.72

Found (%) C 67.41, H 7.62, N 15.78

IR (KBr) νmax (cm^{-1}): 1,699, 1,651 and 1,499

NMR (DMSO-d_6) δ(ppm): 12.97 (s, 1H), 3.95(t, 2H), 3.85 (t, 2H), 2.70–2.60 (m, 1H), 2.35–2.26 (m, 2H), 2.20–2.10 (m, 2H), 1.95–1.82 (m, 4H), 1.80–1.50 (m, 8H), 0.95–0.80 (m, 6H)

^{13}C-NMR (DMSO-d_6) δ (ppm): 159.9, 153.9, 150.7, 147.6, 106.6, 48.8, 48.2, 45.1, 44.2 , 43.2, 41.9, 36.9, 34.1, 20.8, 11.1, 10.9.

EXAMPLE 11

8-(Adamantan-1-yl)methyl-1,3-dipropylxanthine

(Compound 15)

At first 2.00 g (8.85 mmol) of 1,3-dipropyl-5,6-diaminouracil and 2.06 g (10.6 mmol) of 1-adamantan acid were condensed according to the procedure of Example 1 using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide to afford 4.02 g (yield: 100%) of amorphous 6-amino-5-(adamantan-1-yl)acetylamino-1,3-dipropyluracil as a crude product.

NMR ($CDCl_3$) δ(ppm): 7.42 (brs, 1H), 5.47 (brs, 2H), 4.00–3.70 (m, 4H), 2.20–1.20 (m, 21H), 1.10 –0.80 (m, 6H)

The substantially same cyclization reaction as in Example 29 was performed by reacting 3.95 g of the thus obtained compound with phosphorus oxychloride to afford 1.66 g (overall yield: 49%) of the captioned Compound 15 as a white needle crystal.

Melting point: 177.7°–179.5° C. (recrystallized from isopropanol/water)

Elementary analysis: $C_{22}H_{32}N_4O_2$

Calculated (%): C 68.72, H 8.39, N 14.57

Found (%) C 68.71, H 8.74, N 14.70

IR (KBr) νmax (cm^{-1}): 1,704, 1,648 and 1,498

NMR (DMSO-d6) δ(ppm): 13.06 (brs, 1H), 3.95 (t, 2H), 3.83 (t, 2H), 3.40–3.25 (m, 2H), 2.43 (brs, 2H), 1.90 (brs, 3H), 1.80–1.45 (m, 16H), 0.95–0.85 (m, 6H)

EXAMPLE 12

8-[(1IR*,2R*,5S*)-Bicyclo[2.2.1]heptan-2-yl]methyl-1,3-dipropylxanthine

(Compound 16

Compound 16 was obtained in the same procedure as in Example 11, except for using 1.54 ml (10.6 mmol) of (1R*,2R*,5S*)-bicyclo[2.2.1]heptane-2-acetic acid in place of 1-adamantaneacetic acid.

Yield: 1.22 g (White needle crystal; overall yield, 40%)

Melting point: 119.9°–121.4° C. (recrystallized from isopropanol/water)

Elementary analysis: $C_{19}H_{28}N_4O_2$

Calculated (%): C 66.25, H 8.19, N 16.27

Found (%) C 66.29, H 8.32, N 16.06

IR (KBr) νmax (cm^{-1}): 1,699, 1,654 and 1,502

NMR ($CDCl_3$) δ(ppm): 12.83 (brs, 1H), 4.15–4.00 (m, 4H), 2.81 (dd, 1H, J=7.8, 14.2 Hz), 2.66 (dd, 1H, J=7.8, 14.2 Hz), 2.26 (brs, 1H), 2.15–2.00 (m, 2H), 1.95–1.65 (m, 4H), 1.60–1.40 (m, 4H), 1.30–0.90 (m, 10)

EXAMPLE 13

8-[(1R*,4S*, 5S*)-2-Bicyclo[2.2.1]hepten-5-yl]-1,3-dipropyl-7-methylxanthine

(Compound 17)

At first 1.00 g (3.05 mmol) of 8-[(1R*,4S*,5S*)-2-bicyclo[2.2.1]hepten-5-yl]-1 prepared in Example 1 was dissolved in 30 ml of N,N'-dimethylformamide, and 1.05 g carbonate and 0.38 ml (6.10 mmol) of methyl iodide were added thereto. After the mixture was stirring at 50° C. for 30 minutes under argon atmosphere, insoluble materials were filtered off, and the filtrate was concentrated under reduced pressure. The residue was poured into 200 ml of water and the mixture was extracted with chloroform three times. The organic layer was combined, and the extract was washed with water and then with a saturated aqueous sodium chloride, and dried over anhydrous sodium sulfate and then the solvent was evaporated under reduced pressure. The residue was subjected to silica gel column chromatography (eluent: 20% ethyl acetate/hexane) to afford 1.05 g (yield: 100%) of the captioned Compound 17 as a light yellow powder.

Melting point: 99.8°–103.1° C. (recrystallized from acetone/water)

Elementary analysis: $C_{19}H_{26}N_4O_2$

Calculated (%): C 66.64, H 7.65, N 16.36

Found (%) C 66.60, H 7.97, N 16.55

(IR (KBr) νmax (cm^{-1}): 1698, 1666, 1652, 1445.

NMR ($CDCl_3$) δ(ppm): 6.19 (dd, J=3.0, 5.6 Hz, 1H), 5.87 (dd, J=2.8, 5.6 Hz, 1H), 4.01 (t, 2H), 3.95 (t, 2H), 3.94 (s, 3H), 3.36–3.28 (m, 2H), 3.00 (brs, 1H), 2.19 (ddd, J=3.9, 9.3, 11.5 Hz, 1H), 1.84–1.50 (m, 6H), 1.45–1.40 (m, 1H), 1.00–0.90 (m, 6H).

EXAMPLE 14

8-[(1R*,2R*,5R*)-Bicyclo[3.3.0]octan-2-yl]-1,3-dipropyl-7-methylxanthine

(Compound 18)

The substantially same operations as in Example 13 were repeated except for using 1.00 g (2.90 mmol) of 8-[(1R*,2R*,5R*)-bicyclo[3.3.0]octan-2-yl]-1,3-dipropylxanthine (Compound 5) prepared in Example 3 to afford 1.05 g (yield: 100%) of the captioned Compound 18 as a white powder.

Melting point: 94.7°–97.0° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{20}H_{30}N_4O_2$

Calculated (%): C 67.01, H 8.44, N 15.63

Found (%) C 66.93, H 8.20, N 15.68

IR (KBr) νmax (cm^{-1}): 1702, 1653.

NMR ($CDCl_3$) δ(ppm): 4.05 (t, 2H), 3.96 (t, 2H), 3.93 (s, 3H), 2.96–2.84 (m, 1H), 2.80–2.64 (m, 2H), 2.20 –1.60 (m,11H), 1.48–1.25 (m, 3H), 1.02–0.94 (m, 6H).

EXAMPLE 15

1,3-Dipropyl-7-methyl-8-(noradamantan-3-yl)xanthine

(Compound 20)

The substantially same operations as in Example 13 were repeated except for using 2.20 g (6.18 mmol) of

8-(noradamantan-3-yl)-1,3-dipropylxanthine (Compound 14) prepared in Example 10 to afford 1.06 g (yield: 46%) of the captioned Compound 20 as a white needle crystal.

Melting point: 123.2°–124.8° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{21}H_{30}N_4O_2$

Calculated (%): C 68.08, H 8.16, N 15.12

Found (%) C 67.93, H 8.23, N 15.44

IR (KBr) νmax (cm^{-1}): 1698, 1661.

NMR ($CDCl_3$) δ(ppm): 4.05 (t, 2H), 4.01 (s, 3H), 3.96 (t, 2H), 2.98(t, 2H), 2.40 (brs, 2H), 2.25–2.17 (m,2H), 2.11–1.90 (m, 6H).

EXAMPLE 16

7-Ethyl-1,3-dipropyl-8-(noradamantan-3-yl)xanthine

(Compound 21)

The substantially same operations as in Example 13 were repeated except for using 1.40 g (3.93 mmol) of 8-(noradamantan-3-yl)-1,3-dipropylxanthine (Compound 14) prepared in Example 10 and 0.62 ml (7.87 mmol) of ethyl iodide to afford 410 mg (yield: 27%) of the captioned Compound 21 as a white crystal.

Melting Point: 91.3°–92.4° C. (recrystallized from acetonitrile)

Elementary analysis: $C_{22}H_{32}N_4O_2$

Calculated (%): C 68.71, H 8.38, N 14.57

Found (%) C 68.88, H 8.59, N 14.69

IR (KBr) νmax (cm^{-1}): 1698, 1661, 1535.

NMR ($CDCl_3$, 90 MHz) δ(ppm): 4.34 (q, J=7.0 Hz, 2H), 4.20–3.86 (m, 4H), 3.03 (t, 1H), 2.50–1.40 (m, 16H), 1.50 (t, J=7.0 Hz, 3H), 1.15–0.85 (m, 6H).

EXAMPLE 17

3 8-(Noradamantan-3-yl)-1,3,7-tripropylxanthine

(Compound 22)

The substantially same operations as in Example 13 were repeated except for using 1.50 g (4.21 mmol) of 8-(noradamantan-3-yl)-1,3-dipropylxanthine (Compound 14) prepared in Example 10 and 0.82 ml (8.43 mmol) of propyl iodide to afford 1.40 g (yield: 84%) of the captioned Compound 22 as a white crystal.

Melting point: 111.2°–112.2° C. (Recrystallized from ethanol/water)

Elementary analysis: $C_{23}H_{34}N_4O_2$

Calculated (%): C 69.31, H 8.59, N 14.05

Found (%) C 69.29, H 8.69, N 14.57

IR (KBr) νmax (cm^{-1}): 1700, 1662, 1536.

NMR ($CDCl_3$, 90 MHz) δ(ppm): 4.30–3.85 (m, 6H), 3.05 (t, 1H), 2.50–1.50 (m, 18H), 1.20–0.85 (m, 6H).

EXAMPLE 18

8-[(1R*, 2R*, 5R*)-Bicyclo [3.3.0]octan-2-yl]-3-propylxanthine

(Compound 23)

At first, 100 ml of N,N'-dimethylformamide was suspended in 5.00 g (27.2 mmol) of 5,6-diaminouracil [Japanese Published Unexamined Patent Application No. 57,517/80] and 3.88 ml (27.2 mmol) of bicyclo[3.3.0]octan-2-carboxylic acid, 8.40 g (40.8 mmol) of N,N'-dicyclohexylcarbodiimide and then 5.00 g (32.6 mmol) of N-hydroxybenztriazole were added thereto. After the mixture was stirred overnight at room temperature, insoluble materials were filtered off, and the filtrate was concentrated under reduced pressure. 100 ml of aqueous 4N sodium hydroxide solution was added to the residue, and the mixture was refluxed under heating for 20 minutes. After cooling, the mixture was neutralized with concentrated hydrochloric acid, and the precipitated crystals were collected by filtration. 500 ml of water was added to the resulting crystals, and the mixture was extracted with chloroform three times. The combined extract was washed with a saturated aqueous sodium chloride and dried over anhydrous sodium sulfate, and then the solvent was evaporated under reduced pressure. The obtained crude crystals were recrystallized from ethanol-water to afford 3.68 g (yield: 45%) of the captioned Compound 23 as a white needle crystal.

Melting point: 252.8°–257.9° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{16}H_{22}N_4O_2$

Calculated (%): C 63.55, H 7.33, N 18.53

Found (%) C 63.40, H 7.67, N 18.88

IR (KBr) νmax (cm^{-1}): 1700, 1678.

NMR (DMSO-d_6) δ(ppm): 13.03 (brs, 1H), 10.88 (brs, 1H), 3.87 (t, 2H), 2.72–2.50 (m, 3H), 2.10–1.15 (m, 12H), 0.87 (t, 3H).

EXAMPLE 19

8-[(1R*,2R*,5R*)-Bicyclo[3.3.0]octan-2-yl]-1,3-dipropyl-2-thioxanthine

(Compound 24)

The substantially same operations as in Example 1 were repeated except for using 2.00 g (8.26 mmol) of 5,6-diamino-1,3-dipropyl-2-thiouracil [J. Med. Chem., 32, 1873 (1989)] and 1.42 ml (9.92 mmol) of bicyclo[3.3.0]octan-2-carboxylic acid to afford 1.70 g (overall yield: 57%) of the captioned Compound 24 as a white crystal.

Melting point: 135.1°–137.2° C. (recrystallized from ethanol)

Elementary analysis: $C_{19}H_{28}N_4OS$

Calculated (%): C 63.30, H 7.83, N 15.54

Found (%) C 63.54, H 8.14, N 15.59

IR (KBr) νmax (cm^{-1}): 1688, 1493.

NMR ($CDCl_3$) δ(ppm): 12.68 (brs, 1H), 4.68 (t, 2H), 4.63 (t, 2H), 2.92–2.65 (m, 3H), 2.25–1.53 (m, 12H), 1.50–1.43 (m, 1H), 1.41–1.22 (m, 1H), 1.15 −0.98 (m, 6H).

HPLC [AM-312 (15 cm×5 mmφ) (by Yamamura Kagaku K. K.), 70% acetonitrile-water, UV 254 nm, 2.0 ml/min]: Retention time; 14.4 min.

EXAMPLE 20

8-(Noradamantan-3-yl)-3-propylxanthine (Compound 25)

The substantially same operations as in Example 10 were repeated except for using 2.00 g (10.9 mmol) of 1-propyl-5,6-diaminouracil to afford 2.27 g (yield: 63%) of 6-amino-5-(noradamantane-3-carbonylamino)-3-propyluracil as a light yellow powder.

NMR (DMSO-d_6, 90 MHz) δ(ppm): 10.47 (brs. 1H), 7.63 (brs, 1H), 6.23 (brs, 2H), 3.78 (t, 2H), 2.71 (t, 1H), 2.32–1.38 (m, 14H), 0.89 (t, 3H).

The substantially same cyclization reaction as in Example 1 was performed using 2.16 g (6.51 mmol) of the thus obtained compound and 40 ml of a 2N sodium hydroxide aqueous solution to afford 1.85 g (yield: 91%) of the captioned Compound 25 as a white crystal.

Melting point: >290° C. (from dioxane/water)

Elementary analysis: $C_{17}H_{22}N_4O_2$

Calculated (%): C 64.94, H 7.05, N 17.82

Found (%) C 64.65, H 7.20, N 18.00

IR (KBr) νmax (cm^{-1}): 1698, 1660, 1500.

NMR (DMSO-d_6, 90 MHz) δ(ppm): 12.42 (brs, 1H), 11.90 (brs, 1H), 4.08 (t, 2H), 2.77 (t, 1H) 2.55–1.40 (m, 14H), 1.00 (t, 3H).

EXAMPLE 21

8-(Noradamantan-3-yl)-3-propyl-6-thioxanthine

(Compound 26)

At first, 20.0 g (63.7 mmol) of 8-(noradamantan-3-yl)-3-propylxanthine (Compound 25) prepared in Example dissolved in 370 ml of pyridine, and 23.1 g (104 mmol) of phosphorus pentasulfide was added thereto. The mixture was refluxed under heating for 4 hours and poured into ice water, and the solid substances deposited were collected by filtration. The filtrate was concentrated under reduced pressure, and the solid substances deposited were again collected by filtration. The deposited solid substances were combined and dissolved in 200 ml of 2N sodium hydroxide solution, and insoluble materials were filtered off. The filtrate was neutralized with concentrated hydrochloric acid, and the deposited crystals were collected by filtration. The crude crystals were recrystallized from ethanol-water to afford 11.7 g (yield: 56%) of the captioned Compound 26 as a light yellow needle crystal.

Melting point: 214.2°–216.0° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{17}H_{22}N_4OS.1/5H_2O$

Calculated (%): C 61.12, H 6.76, N 16.77

Found (%) C 61.12, H 6.82, N 16.95

IR (KBr) νmax (cm^{-1}): 1668, 1595.

NMR ($CDCl_3$, 90 MHz) δ(ppm): 10.14 (brs, 1H), 9.43 (brs, 1H), 4.05 (t, 2H), 2.73 (t, 1H), 2.68–1.40 (m, 14H), 0.98 (t, 3H).

EXAMPLE 22

8-(Noradamantan-3-yl)-1,3-dimethylxanthine

(Compound 27)

The substantially same operations as in Example 10 were repeated except for using 3.00g (17.6 mmol) of 1,3-dimethyl-5,6-diaminouracil instead of 1,3-dipropyl-5,6-diaminouracil [J. Am. Chem. Soc., 76, 2798 (1954)] to afford 3.61 g (yield: 65%) of 6-amino-5-(noradamantane-3-carbonylamino)-1,3-dimethyluracil as a light yellow powder.

NMR (DMSO-d_6, 90 MHz) δ (ppm): 7.68 (brs, 1H), 6.28 (brs, 2H), 3.30 (s, 3H), 3.11 (s, 3H), 2.71 (t, 1H), 2.66–1.40 (m, 12H).

The substantially same cyclization reaction as in Example 1 was performed except for using 3.60 g (11.3 mmol) of the thus obtained compound to afford 2.41 g (yield: 71%) of the captioned Compound 27 as a white crystal.

Melting point: >295° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{16}H_{20}N_4O_2$

Calculated (%): C 63.98, H 6.71, N 18.65

Found (%) C 63.97, H 6.78, N 18.89

IR (KBr) νmax (cm^{-1}): 1719, 1656, 1649, 1503.

NMR ($CDCl_3$, 90 MHz) δ(ppm): 11.93 (brs, 1H), 3.62 (s, 3H), 3.46 (s, 3H), 2.79 (t, 1H), 2.52–1.60 (m, 12H).

EXAMPLE 23

8-(Noradamantan-3-yl)-1,3-diethylxanthine

(Compound 28)

The substantially same operations as in Example 10 were repeated using 2.0 g (10.1 mmol) of 1,3-diethyl-5,6-diaminouracil [J. Am. Chem. Soc., 75, 114 (1953)9 to afford 2.01 g (yield: 58%) of 6-amino-5-(noradamantane-3-carbonylamino)-1,3-diethyluracil as a light yellow powder

NMR ($CDCl_3$, 90 MHz) δ (ppm): 7.35 (brs, 1H), 5.61 (brs, 2H), 4.18–3.85 (m, 4H), 2.76 (t, 1H), 2.50–1.10 (m, 18H).

The substantially same cyclization reaction as in Example 1 was performed except for using 1.90 g (5.49 mmol) of the thus obtained compound to afford 1.58 g (yield: 88%) of the captioned Compound 28 as a white crystal.

Melting point: 259.8°–263.1° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{18}H_{24}N_4O_2$

Calculated (%): C 65.83, H 7.36, N 17.05

Found (%) C 65.99, H 7.51, N 17.30

IR (KBr) νmax (cm^{-1}): 1704, 1646, 1497.

NMR ($CDCl_3$, 90 MHz) δ (ppm): 11.93 (brs, 1H), 4.40–3.98 (m, 4H), 2.83 (t, 1H), 2.60–1.60 (m, 16H), 1.50–1.18 (m, 6H).

EXAMPLE 24

8-(Noradamantan-3-yl)-1,3-dibutylxanthine

(Compound 29)

The substantially same operations as in Example 10 were repeated except for using 1.70 g (6.69 mmol) of 1,3-dibutyl-5,6-diaminouracil (U.S. Pat. No. 2,607,295) to afford 2.42 g (yield: 90%) of amorphous 6-amino-5-(noradamantane-3-carbonylamino)-1,3-dibutyluracil.

NMR ($CDCl_3$, 90 MHz) δ (ppm): 7.40 (brs, 1H), 5.59 (brs, 2H), 4.05–3.76 (m, 4H), 2.76 (t, 1H), 2.50–0.80 (m, 24H).

The substantially same cyclization reaction as in Example 1 was performed using 2.08 g (5.17 mmol) of the thus obtained compound to afford 1.87 g (yield: 94%) of the captioned Compound 29 as a light yellow powder.

Melting point: 159.7°–161.0° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{22}H_{32}N_4O_2$

Calculated (%): C 68.71, H 8.38, N 14.57

Found (%) C 68.69, H 8.23, N 14.81

IR (KBr) νmax (cm^{-1}): 1704, 1651, 1498.

NMR ($CDCl_3$, 90 MHz) δ (ppm): 11.67 (brs, 1H), 4.28–3.90 (m, 4H), 2.82 (t, 1H), 2.62–1.19 (m, 18H), 1.15–0.80 (m, 6H).

EXAMPLE 25

8-(Noradamantan-3-yl)-3-isobutyl-1-methylxanthine

(Compound 30)

The substantially same operations as in Example 10 were repeated except for using 1.87 g (8.81 mmol) of 1-isobutyl-3-methyl-5,6-diaminouracil [Methods in Enzymology, 159, 489 (1988)] to afford 2.63 g (yield: 83%) of 6-amino-5-(noradamantane-3-carbonylamino)-1-isobutyl -3methyluracil as a white powder.

NMR ($CDCl_3$, 90 MHz) δ (ppm): 7.35 (brs, 1H), 5.56 (brs, 2H), 3.77 (d, J=7.7 Hz, 2H), 3.34 (s, 3H), 2.76 (t, 1H), 2.40–1.50 (m, 13H), 0.99 (d, J=6.6 Hz, 6H)

The substantially same cyclization reaction as in Example 1 was performed except for using 2.60 g (7.21 mmol) of the thus obtained compound to afford 1.69 g (yield: 68%) of the captioned Compound 30 as a white needle crystal.

Melting point: 266.0°–268.7° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{19}H_{26}N_4O_2$

Calculated (%) C 66.64, H 7.65, N 16.36

Found (%) C 66.89, H 7.44, N 16.42

IR (KBr) νmax (cm^{-1}): 1708, 1652, 14

NMR ($CDCl_3$, 90 MHz) δ (ppm) 11.72 (brs, 1H), 3.98 (d, J=7.5 Hz, 2H), 3.44 (s, 3H), 2.77 (t, 1H), 2.52–1.60 (m, 13H), 0.95 (d, J=6.6 Hz, 6H).

EXAMPLE 26

8-(Noradamantan-3-yl)-1,3-dipropyl-2-thioxanthine

(Compound 31)

The substantially same operations as in Example 10 were repeated except for using 3.00 g (12.4 mmol) of 5,6-diamino-1,3-dipropyl-2-thiouracil and 2.27 g (13.6 mmol) of noradamantane-3-carboxylic acid to afford 2.95 g (yield: 60%) of amorphous 6-amino-5-(noradamantane-3-carbonylamino)-1,3-dipropyl-2-thiouracil.

NMR ($CDCl_3$, 90 MHz) δ (ppm): 7.50 (brs, 1H), 5.80 (brs, 2H), 4.60–4.25 (m, 4H), 2.72 (t, 1H), 2.40–1.50 (m, 16H), 1.20–0.80 (m, 6H).

The substantially same cyclization reaction as in Example 29 was performed except for using 2.70 g (6.92 mmol) of the thus obtained compound instead of 6-amino-5-(adamantane-1-carbonylamino)-1,3-dipropyluracil to afford 765 mg of the captioned Compound 31 as a light yellow powder.

Melting point: 216.2°–216.6° C. (recrystallized from isopropanol)

Elementary analysis: $C_{20}H_{28}N_4OS$

Calculated (%) C 64.48, H 7.58, N 15.04

Found (%) : C64.49, H 7.56, N 15.35

IR (KBr) νmax (cm^{-1}): 1690, 1494.

NMR ($CDCl_3$) δ (ppm): 11.96 (brs, 1H), 4.69 (t, 2H), 4.61 (t, 2H), 2.86 (t, 1H), 2.48–2.42 (m, 2H), 2.35–2.26 (m, 2H), 2.15–1.85 (m, 12H), 1.15–0.95 (m, 6H).

Example 27

8-(Noradamantan-3-yl)-1,3-dipropyl-6-thioxanthine

(Compound 32)

The substantially same operations as in Example 21 were repeated except for using 2.00 g (5.62 mmol) of 8-noradamantan-3-yl)-1,3-dipropylxanthine (Compound 14in Example 10 to afford 2.02 g (yield: 70%) of the captioned Compound 32 as a light yellow crystal.

Melting point: 128.5°–130.4° C. (recrystallized from acetonitrile)

Elementary analysis: $C_{20}H_{28}N_4OS$

Calculated (%): C 64.48, H 7.58, N 15.04

Found (%) C 64.49, H 7.66, N 15.29

IR (KBr) νmax (cm^{-1}): 1,682, 1,597 and 1,495

NMR ($CDCl_3$, 90 MHz) δ (ppm): 9.65 (brs, 1H), 4.43 (t, 2H), 4.06 (t, 2H), 2.69 (t, 1H), 2.53–1.60 (m, 16H), 1.10–0.85 (m, 6H).

EXAMPLE 28

8-(Noradamantan-3-yl)-1,3-dipropyl-2,6-dithioxanthine

(Compound 33)

The substantially same operations as in Example 21 were repeated except for using 2.00 g (5.38 mmol) of 8-noradamantan-3-yl)-1,3-dipropyl-2-thioxanthine (Compound 31) prepared in Example 26 to afford 1.27 g (yield: 61%) of the captioned Compound 33 as a light yellow powder.

Melting point: 94.2°–96.6° C. (recrystallized from acetonitrile)

Elementary analysis: $C_{20}H_{28}N_4S_2 0.1CH_3CN 0.2H_2O$

Calculated (%): C 61.22, H 7.30, N 14.49

Found (%) C 61.18, H 7.38, N 14.57

IR (KBr) νmax (cm^{-1}): 1604, 1504, 1088.

NMR ($CDCl_3$, 90 MHz) δ (ppm): 9.46 (brs, 1H), 5.06 (t, 2H), 4.62 (t, 2H), 2.72 (t, 1H), 2.53–1.55 (m, 16H), 1.15–0.85 (m, 6H).

Example 29

8-(Adamantan-1-yl)-1,3-dipropylxanthine

(Compound 13)

At first, 10 g (44.3 mmol) of 1,3-dipropyl-5,6-diaminouracil was dissolved in 50 ml of pyridine, and 10.6 g (53.1 mmol) of adamantane-1-carbonylchloride was added by portions thereto at 0° C. After stirring for 30 minutes at 0° C., the mixture was concentrated under reduced pressure. A saturated aqueous sodium bicarbonate was added thereto. The residue was extracted with chloroform three times. The organic layers were combined, washed with a saturated aqueous sodium chloride, and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. Then pyridine was removed from the residue by means of azeotropy with toluene to afford 19.5 g (yield: 100%) of 6-amino-5-(adamantane-1-carbonylamino)-1,3-dipropyluracil.

NMR (90MHz; $CDCl_3$) δ (ppm): 7.47 (brs, 1H), 5.60 (brs, 2H), 4.05–3.70 (m, 4H), 2.25–1.45 (m, 19H), 1.15 –0.85 (m, 6H)

Then, 100 ml of phosphorus oxychloride was added to 19.5 g of the thus obtained compound and the mixture was refluxed under heating for 30 minutes. The mixture was concentrated under reduced pressure, and a saturated aqueous sodium bicarbonate was added to the residue. The residue was extracted with chloroform three times. The extract was dried over anhydrous sodium sulfate and then the solvent was evaporated under reduced pressure. The residue was subjected to silica gel column chromatography (eluent: 20% ethyl acetate/hexane) and recrystallized from isopropanol-water to afford 2.07 g (overall yield: 13%) of the captioned Compound 13 as a white needle crystal.

Melting point: 169.3°–171.0° C.

Elementary analysis: $C_{21}H_{30}N_4O_2$

Calculated (%): C 68.08, H 8.16, N 15.12

Found (%) C 68.10, H 8.30, N 15.09

IR (KBr) νmax (cm^{-1}): 1699, 1650, 1491

NMR ($CDCl_3$) δ (ppm): 11.70 (brs, 1H), 4.15–3.95 (m, 4H), 2.15–2.05 (m, 9H), 1.85–1.50 (m, 10H), 1.05 –0.85 (m, 6H)

EXAMPLE 30

8-(Adamantan-1-yl)-1,3-dipropyl-7-methylxanthine

(Compound 19)

The substantially same operations as in Example 13 were repeated except for using 2.50 g (6.76 mmol) of 8-(adamantan-1-yl)-1,3-dipropylxanthine (Compound 13) prepared in Example 29 to afford 2.16 g (yield: 83%) of the captioned Compound 19 as a white crystal.

Melting point: 79.8°–80.9° C. (recrystallized from ethanol/water)

Elementary analysis: $C_{22}H_{32}N_4O_3$

Calculated (%): C 68.17, H 8.38, N 14.57

Found (%) C 68.28, H 8.47, N 14.72

IR (KBr) νmax (cm^{-1}): 1698, 1659.

NMR (DMSO-d_6) δ (ppm): 4.10 (s, 3H), 3.93 (t, 2H), 3.82 (t, 2H), 2.13–2.02 (m, 9H), 1.82–1.46 (m, 10H), 0.90–0.80 (m, 6H).

EXAMPLE 31

1,3-Diallyl-8-(3-noradamantyl)xanthine

(Compound 34)

At first, 1.65 g (9.91 mmols) of 3-noradamantanecarboxylic acid was dissolved in 20 ml of pyridine, and 0.80 ml (10.8 mmols) of thionyl chloride was added dropwise thereto at 0° C. The mixture was stirred for 1 hour at room temperature, and cooled to 0° C. once again. 2.00 g (9.01 mmols) of 5,6-diamino-1,3-diallyluracil [NaunynSchmiedeberg's Arch. Pharmacol., 336, 204 (1987)] in 20 ml of a methylene chloride solution was added dropwise. The reaction mixture was further stirred for 1 hour at room temperature, and concentrated under reduced pressure. The concentrate was poured into 100 ml of water. The mixture was extracted with 30 ml of chloroform three times. The organic layers were combined, washed with a saturated aqueous sodium chloride, and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. The residue was purified and isolated by silica gel column chromatography (eluent: 2% methanol/-chloroform) to afford 2.44 g (yield: 73%) of 6-amino-1,3-diallyl-5-(noradamantane-3-carbonylamino)uracil as amorphous.

NMR (90 MHz; $CDCL_3$)δ(ppm): 7.41 (1H, brs), 6.20–5.10 (8H, m), 4.80–4.45 (4H, m), 2.76 (1H, t), 2.50–1.50 (12H, m).

Then, 27 ml of 2N sodium hydroxide aqueous solution and 14 ml of dioxane were added to 2.29 g (6.19 mmols) of the obtained compound and the mixture was refluxed under heating for 1 hour. After cooling, the reaction mixture was neutralized with concentrated hydrochloric acid, and extracted three times with chloroform. Then the organic layers were combined, washed with a saturated aqueous sodium chloride and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. The obtained crude crystals were recrystallized from isopropanol-water to afford 1.55 g (yield: 71%) of Compound 34 as a white needle crystal.

Melting point: 202.0–204.3° C

Elemental analysis: $C_{20}H_{24}N_4O_2$

Calculated (%): C68.16, H 6.86, N 15.89

Found (%): C 68.20, H 6.97, N 15.63

IR (KBr) νmax (cm^{-1}): 3190, 2940, 1704, 1658, 1651, 1550, 1498

NMR 90 MHz; $CDCl_3$)δ(ppm): 6.30–5.70 (2H, m), 5.60–5.00 (4H, m), 4.90–4.60 (4H, m), 2.80 (1H, t), 2.55–1.50 (12H, m)

MS (m/e): 352 (M+).

EXAMPLE 32

3-Allyl-8-(3-noradamantyl)-1-propargylxanthine

Compound 35

At first, 1.10 g (3.21 mmols) of 3-allyl-8-(3-noradamantyl)xanthine obtained in reference Example 1 was dissolved in 32 ml of dimethylformamide, and 257 mg (6.42 mmols) of 60% sodium hydride was added at 0° C. After the mixture was stirred for 30 minutes at room temperature, 0.25 ml (3.36 mmols) of propargyl bromide was dropwise added thereto. The mixture was stirred for 4 hours at room temperature, and the reaction mixture was poured into 300 ml of water and was neutralized with 1N hydrochloric acid. The mixture was extracted with 50 ml of chloroform three times. The organic layers were combined, washed with water twice and then with a saturated aqueous sodium chloride, and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. The residue was purified and isolated by flush chromatography (eluent: 40% ethyl acetate/hexane) and recrystallized from dimethysulfoxide-water to afford 110 mg (yield: 23%) of Compound 35 as a white powder.

Melting point: 256.3–257.1° C.

Elemental analysis: $C_{20}H_{22}N_4O_2$

Calculated (%): C 68.55, H 6.32, N 15.98

Found (%): C 68.62, H 6.45, N 16.05

IR (KBr) νmax (cm^{-1}): 1700, 1659, 1649, 1548, 1496

NMR (270 MHz; $CDCl_3$) δ(ppm): 11.58 (1H, brs), 6.10–5.90 (1H, m), 5.45–5.20 (2H, m), 4.90–4.70 (4H, m), 2.80 (1H, t), 2.50–1.90 (8H, m), 1.80–1.60 (5H, m).

MS (m/e): 350 (M+).

REFERENCE EXAMPLE 1

3-Allyl-8-(3-noradamantyl)xanthine

At first, 3.22 g (19.4 mmols) of 3-noradamantanecarboxylic acid was dissolved in 80 ml of pyridine, and 1.54 ml (21.1 mmols) of thionyl chloride was dropwise added under ice-cooling. The mixture was stirred for 50 minutes at room temperature. 3.21 g (17.6 mmols) of 1-allyl-5,6-diaminouracil (U.S. Pat. No. 2,673,848) was gradually added to the reaction mixture under ice-cooling. After stirring for 2 hours at room temperature, the reaction mixture was concentrated under reduced pressure. The residue was extracted five times with chloroform/methanol (5/1). The organic layers were combined and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. 30 ml of dioxane and 60 ml of aqueous 1N sodium hydroxide solution were added to the residue, and the mixture was refluxed under heating for 30 minutes. After cooling, the reaction mixture was neutralized with 1N hydrochloric acid, and was extracted three times with 50 ml of chloroform. The organic layers were combined, washed with a saturated aqueous sodium chloride and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to afford 4.92 g (yield: 90%) of the captioned Compound as a light yellow plate crystal.

Melting point: >270° C. (recrystallized from ethanol/water)

Elemental analysis: $C_{17}H_{20}N_4O_2$

Calculated (%): C 65.36, H 6.45, N 17.93

Found (%): C 64.98, H 6.72, N 17.86

IR (KBr) νmax (cm^{-1}): 1685, 1648, 1643, 1498, 1425

NMR (90 MHz; $CDCl_3$) δ(ppm): 12.10 (1H, brs), 7.20 (1H, s), 6.20–5.65 (1H, m), 5.45–5.05 (2H, m), 4.80–4.45 (2H, m), 2.71 (1H, t), 2.55–1.50 (12H, m).

Pharmaceutical Preparation 1

Tablet

A tablet having the following composition was prepared according to the conventional method

Compound 3	20 mg
Lactose	60 mg
Potato starch	30 mg
Polyvinyl alcohol	3 mg
Magnesium stearate	1 mg

Pharmaceutical Preparation 2

Powder

A powder having the following composition was prepared according to the conventional method.

Compound 1	20 mg
Lactose	300 mg

Pharmaceutical Preparation 3

Syrup

A syrup having the following composition was prepared according to the conventional method.

Compound 2	20 mg
Refined saccharose	30 mg
Ethyl p-hydroxybenzoate	40 mg
Propyl p-hydroxybenzoate	10 mg
Strawberry flavor	0.1 ml
Water to make the total volume	100 ml

Pharmaceutical Preparation 4

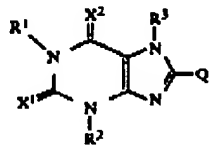
Capsule

Ingredients set forth below were admixed and charged into gelatin capsules in accordance with the conventional method to thereby prepare a capsule.

Compound 3	20 mg
Lactose	200 mg
Magnesium stearate	5 mg

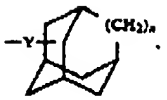
What is claimed is:

1. Xanthine compounds represented by the following formula (I):



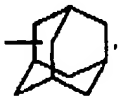
(I)

wherein each of X^1 and X^2 independently represents oxygen or sulfur; and Q represents:



where Y represents a single bond or alkylene having 1 to 4 carbon atoms, n represents 0 or 1,

each of R^1 and R^2 independently represents hydrogen, lower alkyl, allyl or propargyl, and R^3 represents hydrogen or lower alkyl, provided that when Q is



then

R^1, R^2 and R^3 are not simultaneously methyl; or a pharmaceutically acceptable salt thereof.

2. The compound according to claim 1, wherein both of R^1 and R^2 are lower alkyl and R^3 is hydrogen; and both of X^1 and X^2 are oxygen.

3. The compound according to claim 1, wherein each of R^1, R^2 and R^3 independently represents hydrogen or lower alkyl.

4. The compound according to claim 1, wherein each of R^1 and R^2 independently represents allyl or propargyl and R^3 represents hydrogen or lower alkyl.

5. The xanthine compound according to claim 4, wherein X^1 and X^2 are both oxygen and n is 0.

6. The compound according to claim 3, wherein Q

7. The compound according to claim 3, wherein Q

8. 8-(Noradamantan-3-yl)-1,3-dipropylxanthine or a pharmaceutically acceptable salt thereof.

9. 1,3-Diallyl-8-(3-noradamantyl)xanthine, or a pharmaceutically acceptable salt thereof.

10. 3-Allyl-8-(3-noradamantyl)-1-propargylxanthine, or a pharmaceutically acceptable salt thereof.

11. A pharmaceutical composition comprising a pharmaceutical carrier and, as an active ingredient, an effective amount of a xanthine compound as defined by claim 1 or a pharmaceutically acceptable salt thereof.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL. Page 1 of 8

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page: Item

AT [56] REFERENCES CITED

Other Publications,
"Shamin et al. J. Med. Chem., (1989) vol. 32, 1231."
should read
--Shamin et al., J. Med. Chem., (1988) vol. 31, 613.--.

AT [57] ABSTRACT

Line 1, "where -----" should read -- where ----- --.
Line 5, "-NH-; represants" should read
-- -NH-; when Q represents--.

COLUMN 2

Line 14, "where -----" should read -- where ----- --.
Line 47, Close up right margin.
Line 48, Delete blank line.
Line 49, "methyl;" should read --methyl; [hereinafter--.
Line 60, "etc" should read --etc.--.

COLUMN 5

Line 28, "dimethylformamide" should read
--dimethylformamide,--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL. Page 2 of 8

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 15

Line 21, Close up right margin.
Line 22, Close up left margin.
Line 23, "effect" should read --effect,--.
Line 58, "HCl" should read --HCl.--.

COLUMN 16

Line 48, "equation. 2)Adenosine" should read --equation. ¶ 2)Adenosine--.

COLUMN 20

Line 10, "24 h" should read --24 hours--.

COLUMN 23

Line 66, "unit" should read --units--.

COLUMN 25

Line 26, "N 16, 78" should read --N 16.78--.
Line 62, "25," should be deleted.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL. Page 3 of 8

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 26

Line 16, "6(ppm):" should read --δ(ppm):--.
Line 52, "and" should read --and 8-[(1R*,2S*,5R*)- --.

COLUMN 28

Line 4, "(m, 6H) 8-(Benzo[b]furan-" should read
--(m, 6H)
Example 7
8-(Benzo[b]furan- --.
Line 15, Close up right margin.
Line 23, "Compound 11)" should read --(Compound 11)--.
Line 39, "(Compound 12" should read --(Compound 12)--.
Line 45, "6(ppm):" should read --δ(ppm):--.

COLUMN 29

Line 19, "6(ppm):" should read --δ(ppm):--.
Line 28, "1-adamantan" should read
--1-adamantaneacetic--.
Line 45, "Found (%)" should read --Found (%):--.
Line 47, "6(ppm):" should read --δ(ppm):--.
Line 53, "8-[(11R*," should read --8-[(1R*,--.
Lind 55, "(Compound 16" should read --(Compound 16)--.
Line 66, "Found (%)" should read --Found (%):--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL. Page 4 of 8

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 30

Line 1, "6(ppm):" should read --δ(ppm):--.
Line 13, "hepten-5-yl]-1 prepared" should read --hepten-5-yl]-1, 3-dipropylxanthine (Compound 1) prepared--.
Line 15, "g carbonate" should read --g (7.61 mmol) of potassium carbonate--.
Line 34, "Found (%)" should read --Found (%):--.
Line 57, "Found (%)" should read --Found (%):--.
Line 59, "6(ppm):" should read --δ(ppm):--.

COLUMN 31

Line 8, "Found (%)" should read --Found (%):--.
Line 10, "6(ppm):" should read --δ(ppm):--.
Line 28, "Found (%)" should read --Found (%):--.
Line 35, "3 8-(Noradamantan-3-yl)-" should read --8-(Noradamantan-3-yl)- --.
Line 48, "Found (%)" should read --Found (%):--.
Line 50, "6(ppm):" should read --δ(ppm):--.

COLUMN 32

Line 18, "Found (%)" should read --Found (%):--.
Line 20, "6(ppm):" should read --δ(ppm):--.
Line 39, "Found (%)" should read --Found (%):--.
Line 50, "-3-propylxanthine (Com-" should read -- -3-propylxanthine ¶ (Com- --.
Line 57, "$_{90}$ MHz" should read --90 MHz--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL. Page 5 of 8

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 33

Line 1, "Found (%)" should read --Found (%):--.
Line 2, "υmax (cm^{-bi}):" should read --υmax (cm^{-1}):--.
Line 3, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.
Line 11, "Af" should read --At--.
Line 12, "Example" should read --Example 20--.
Line 34, "Found (%)" should read --Found (%):--.
Line 36, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.
Line 51, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.
Line 65, "Found (%)" should read --Found (%):--.
Line 67, "$_{90}$ MHz" should read --90 MHz--.

COLUMN 34

Line 8, "(1953)9" should read --(1953)]--.
Line 11, "powder" should read --powder.--.
Line 12, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.
Line 24, "Found (%)" should read --Found (%):--.
Line 26, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.
Line 39, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.
Line 50, "Found (%)" should read --Found (%):--.
Line 52, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.
Line 65, "$_{90}$ MHz) δ(ppm):" should read --90 MHz) δ(ppm):--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 35

Line 9, "Calculated (%)" should read --Calculated (%):--.
Line 10, "Found (%)" should read --Found (%):--.
Line 11, "14" should read --1495--.
Line 12, "6(ppm)" should read --δ(ppm):--.
Line 13, "(t, 1H)," should read --(t, 1H),--.
Line 25, "6(ppm):" should read --δ(ppm):--.
Line 32, "765 mg of" should read
--765 mg (yield: 30%) of--.
Line 37, "Calculated (%)" should read --Calculated (%):--.
Line 40, "6(ppm):" should read --δ(ppm):--.
Line 51, "14 in" should read --14) prepared in--.
Line 57, "Found (%)" should read --Found (%):--.
Line 59, "6(ppm):" should read --δ(ppm):--.

COLUMN 36

Line 11, "6(ppm):" should read --δ(ppm):--.
Line 34, "6(ppm):" should read --δ(ppm):--.
Line 54, "Found (%)" should read --Found (%):--.
Line 56, "6(ppm):" should read --δ(ppm):--.
Line 58, "6H)" should read --6H).--.

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CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL. Page 7 of 8

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 37

Line 5, "Found (%)" should read --Found (%):--.
Line 7, "δ(ppm):" should read --δ(ppm):--.
Line 35, "$CDCL_3$)δ(ppm):" 7.41" should read --$CDCL_3$) δ(ppm):" ¶ 7.41--.
Line 57, "$CDCl_3$)δ(ppm): 6.30-5.70" should read --$CDCl_3$) δ(ppm): ¶ 6.30-5.70--.
Line 58, "(4H, m), 4.90-4.60" should read --(4H, m), ¶ 4.90-4.60--.

COLUMN 38

Line 23, "(2H, m), 4.90-4.70" should read --(2H, m), ¶ 4.90-4.70--.
Line 68, "method" should read --method.--.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,290,782

DATED : March 1, 1994

INVENTOR(S) : FUMIO SUZUKI ET AL. Page 8 of 8

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 40

Line 28, Close up right margin.
Line 35, "Q" should read --Q is--.
Line 42, "Q" should read --Q is--.

Line 37, "  " should read --  --.

Signed and Sealed this

Sixteenth Day of May, 1995

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

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JOURNAL OF Dermatological Science

Dry condition affects desquamation of stratum corneum in vivo

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Abstract

We examined whether a dry condition actually induces scaly skin in vivo. Hairless mice were kept in a high humidity condition or a low humidity condition and skin changes were examined. Scales appeared on the backs of mice kept for 3 days under the dry condition. The weight of stratum corneum (SC) was increased at this point, and these alterations were not accompanied with hyperproliferation of the nucleated cell layer of the epidermis. A decrease of desmosomal degradation was observed, though, desquamation-related enzyme activity was not altered. The regulation mechanism of desquamation is not yet clear, however, in vitro experiments suggest that the water content in SC is an important factor. The water content of SC was decreased in the dry condition. These results indicate that a dry environment perturbs desmosome degradation in intact SC by decreasing the water content of SC, and the consequent impairment of desquamation in normal skin in vivo may lead to the induction of a scaly skin surface.

Keywords: Desmosomes; Scale; Humidity

1. Introduction

The most common skin problem in humans of all age groups is dry, rough and flaky skin. Although details of the mechanism inducing this problem are not fully understood yet, it is clear that alterations in stratum corneum (SC) desquamation are involved.

Lundström and Egelrud [1,2] showed the existence of desmoglein I, a characteristic transmembrane protein of desmosomes, in plantar SC, which suggests that desmosomes should take part in cohesion of corneocytes. They also found a chymotrypsin-like serine protease which is stored in lamellar bodies in the stratum granulosum until it functions within the SC extracellular spaces [3,4]. In addition to the chymotrypsin-like enzyme, we found a trypsin-like protease in normal SC [5,6]. Topical application of serine-type protease inhibitors induced scale, supporting that these proteases are actually functional in vivo [7].

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It is still unclear what factors regulate desquamation of SC in vivo. The results from an in vitro experiment, suggests that a high content of water promotes degradation of desmosomes, that the water content of the SC is one of important factors [8]. Furthermore, observations that many scaling diseases are worsened when climatic conditions become cold and dry [9–11], support this idea. To elucidate this possibility, we examined whether or not a dry condition actually induces scaly skin by assessing the number of SC scales on hairless mice kept under high or low humidity conditions for 7 days. We also traced the changes in histological appearance, desmosomal protein, SC water content and protease activities in the SC of these animals.

2. Materials and methods

2.1. Animals and conditions

Eight- to ten-week-old male hairless mice (Hos: HR-1) were obtained from Hoshino, Japan. Before each experiment, the animals were kept in individual cages for at least 3 weeks. These cages were placed in the same room with the temperature maintained at 22–25°C and relative humidity at 40–50%. The animals were assigned to two groups. One group was exposed to a dry condition and the other to a moist condition. The dry group animals were kept in a 7.2-l cage in which the relative humidity was maintained at 10%, while the moist group animals were kept in the same size cage in which the relative humidity was kept above 80%. The temperature was maintained at 22–25°C. Fresh air was circulated 100 times per hour, but the animals were not directly exposed to the air stream. The level of NH_3 was always kept below 1 ppm. After 0, 1, 2, 3, 4 and 7 days, biopsy and collection of SC by tape-stripping were undertaken.

2.2. Skin surface appearance

The skin surface was observed on the backs of the hairless mice with a Micro Hi-Scope System (Hirox, Japan). The number of scales was counted in binary images [7].

2.3. Histologic observation and SC weight determination

Biopsy samples were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Measurements of the thickness of the epidermis were made with a light microscope equipped with a CCD camera and image analysis system (Olympus XL-10, Japan). SC sheets were obtained by trypsin treatment [12]. In order to calculate the thickness of SC, the weight per area of SC sheets was measured.

2.4. Electron microscopic observation

Biopsy samples were fixed in half-strength Karnovsky's fixative, divided and processed through reduced 1.0% osmium tetroxide, then embedded in Epon-epoxy mixture. Ultrathin sections were viewed in an electron microscope (H7100, Hitachi, Japan) after further contrasting in lead citrate and uranyl acetate.

2.5. Analysis of desmosomal protein

Desmosomal proteins were extracted from tape-stripped SC in a buffer containing 0.1 M Tris–HCl (pH 9), 9 M urea, 2% SDS, and 1% mercaptoethanol, (200 μl of buffer per 2 mg SC) for 15 h at 37°C [4]. The extracts were mixed with a Laemmli's sample buffer, and heated on a boiling water bath for 10 min. After centrifugation, the supernatant was analyzed by SDS–PAGE in 10% gel. Electophoretic transfer of proteins from the gel to PVDF membrane (Applied Biosystems, USA) was followed by immunostaining with monoclonal antibody, anti-DG I (Boehringer Mannheim, Germany), against the desmosomal glycoprotein, desmoglein I, using a Promega Protoblot Western Blot AP System (Promega, USA).

2.6. Enzyme activity

Trypsin-like enzyme activity of SC was examined by using Boc-Phe-Ser-Arg-MCA (Peptide Institute, Japan) as the substrate [5,6]. All assays were performed at 37°C in 0.1 M Tris–HCl (pH 8.0).

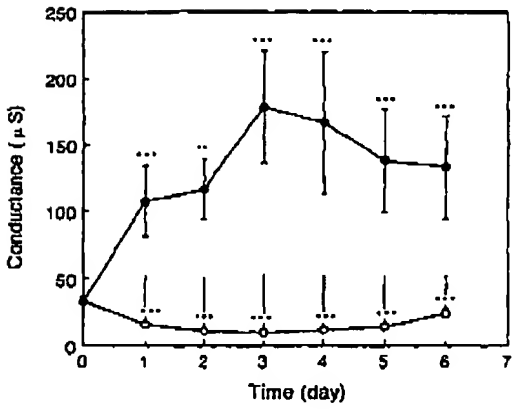

Fig. 1. The changes of skin conductance measured with a Skicon-200. Skin conductance decreased in the dry condition (○), and increased in the moist condition (●; $n = 6$, mean ± S.E.M.). Significances versus day 0 ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

2.7. Conductance

Conductance of the back of hairless mice was measured with a Skicon-200 high-frequency hygrometer (IBS, Hamamatsu, Japan) [13].

2.8. Statistics

The significance of differences was tested by using the Mann–Whitney *U*-test.

3. Results

3.1. Changes in water content of SC

At first, we examined whether or not the dry or the moist condition affects the water content of SC in vivo. In the dry condition, the value of skin conductance was decreased on and after day 1 (Fig. 1). On the other hand, the value of skin conductance was increased from day 1 in the moist condition. These results show that the dry condition lowers the water content of SC in vivo, while the moist condition increases it.

3.2. Changes of skin surface and SC in the dry condition

Many scales were observed on the backs of mice exposed for 3 days to dry versus normal condition (Fig. 2(b) vs. Fig. 2(a)). The number of scales was increased on and after 3 days in the dry condition (Fig. 3(a)). Biopsy results showed that the dry weight of SC also increased on and after 3 days in the dry condition (Fig. 4(a)). The thickness of the nucleated cell layer of the epidermis was quantified and it had not changed statistically for 3 days in the dry condition (data not shown).

These results show that the dry condition induces scales on the skin surface and increases the thickness of SC without inducing epidermal hyperplasia.

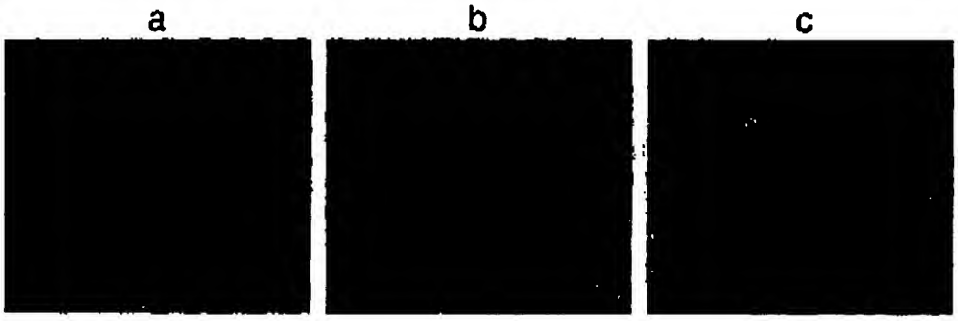

Fig. 2. The skin surface appearance on the backs of mice kept in conditions of different humidity. More scales were observed after 3 days in the dry condition (b) than in the normal condition (a). Scales were decreased after 3 days in the moist condition (c) as compared with the normal condition (a).

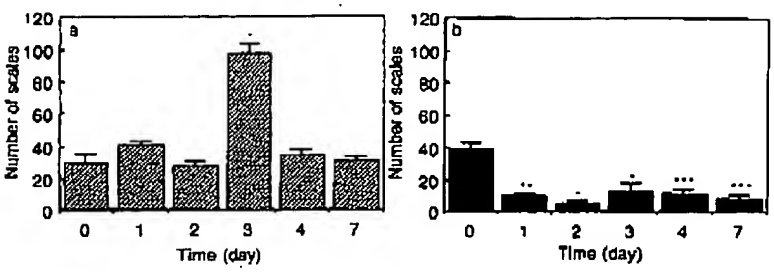

Fig. 3. (a) The number of scales was increased on and after 3 days in the dry condition ($n = 3$, mean ± S.E.M.). (b) The number of scales was decreased on and after day 1 in the moist condition ($n = 3$, mean ± S.E.M.). Significances versus day 0 ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

3.3. Changes of skin surface and SC in the moist condition

The number of scales was decreased on and after the first day in the moist condition (Fig. 2(c) and Fig. 3(b)). However, biopsy results showed that the dry weight of SC was not changed (Fig. 4(b)). The thickness of the nucleated cell layer of the epidermis remained unchanged statistically (data not shown).

Thus, the moist condition decreased scale on the skin surface, but did not affect the weight of the SC or the thickness of the nucleated cell layer of the epidermis.

3.4. Desmosomes

In the normal condition, corneocytes showed a piled-up ultrastructure. Desmosomes remained in the upper SC at the edges of corneocytes, but desmosomes were degraded from the middle to the upper layer of SC at the center of corneocytes (Fig. 5(a)). On the other hand, at the center of corneocytes after 3 days in the dry condition, desmosomes were also seen in the intercellular space, even in the upper layers of the SC (Fig. 5(b)). As shown in Fig. 6, the content of desmoglein I in SC increased after 3 days in the dry condition. There were no significant changes of the desmoglein I content in SC between the normal and the moist group of mice.

These results show that desmosomes are less effectively degraded in the dry condition.

3.5. Changes of enzyme activity related to desquamation in the dry condition

We previously reported that digestion of desmosomes by two types of serine proteases lead to desquamation [5,6]. Enzyme activity in SC obtained by tape-stripping was examined. Trypsin-like enzyme activity in the SC from animals which had been kept in the dry condition showed no change (Table 1).

4. Discussion

Under conditions of low humidity in winter, scaly skin occurs not only in pathological lesions, but also in normal skin. Patients with cutaneous xerosis show an increased number of scales, and more severe symptoms are seen in aged people [11]. Chernosky [14,15] also reported that dry skin occurs in winter. Kligman et al. [16] reported that the SC thickness of xerosis vulgaris on the leg increases in winter. Further, desmosomes are retained in the surface layers of the SC in winter xerosis [17]. These observations suggest a link between dry conditions and abnormal desquamation in SC.

In vitro studies have shown that environmental humidity influences the water content of SC [13,18], which decreases gradually from 75 to 30% in relative humidity [13]. These reports suggested that the water content of SC was great importance in maintaining good skin condition [18]. Rawlings et al. [8] showed in their in vitro studies

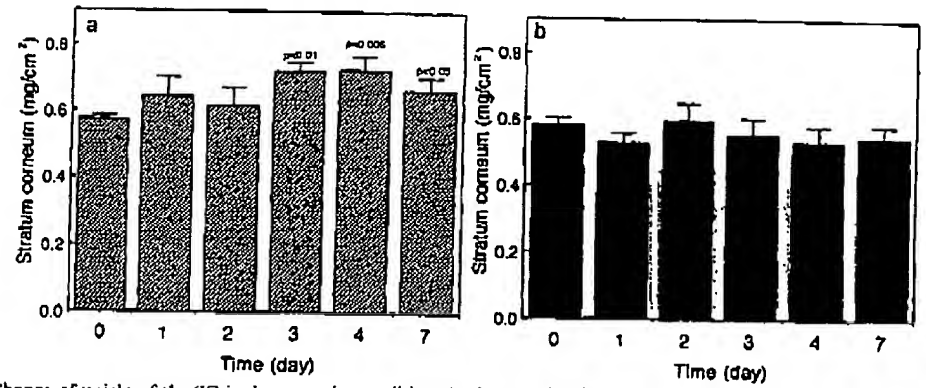

Fig. 4. Change of weight of the SC in dry or moist condition. (a) Increased weight of the SC from mice kept in the dry condition on and after 3 days ($n = 4–7$, mean ± S.E.M.). There were no change up to 7 days in the moist condition ($n = 4–8$, mean ± S.E.M.). Significances versus day 0.

that a dry condition inhibits desmosomal degradation as compared to a moist condition. These results suggest that water content in SC is an important factor for desmosomal degradation.

In the present study, we found that many scales appeared on the backs of mice kept for 3 days in the dry condition. The thickness of SC was increased, and these alterations occurred without

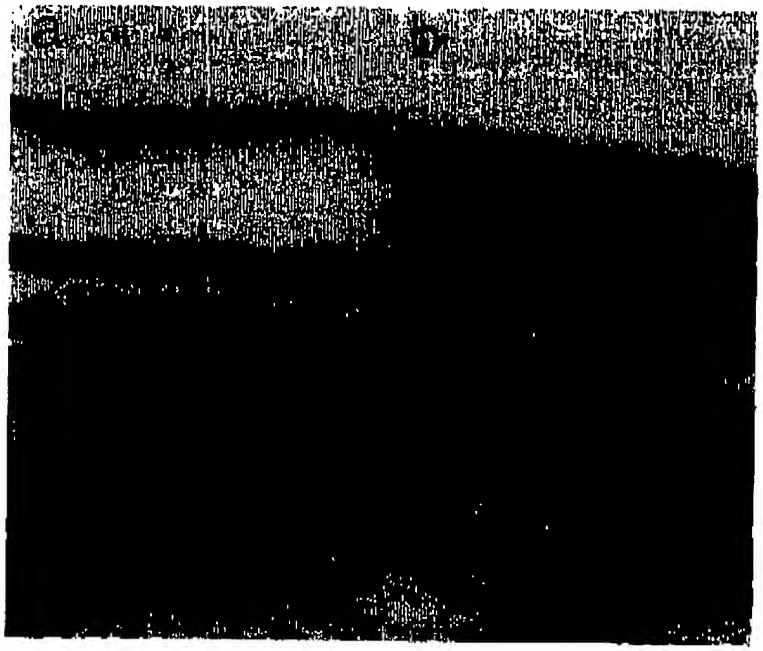

Fig. 5. Ultrastructure of SC from mice kept in the dry condition and normal condition. At the center of corneocytes, desmosomes (arrows) were degraded from the middle to the upper layer of SC in normal conditions (a). After 3 days in the dry condition, desmosomes (arrows) remined in the upper layers (b). Scale bar, 1.0 μm.

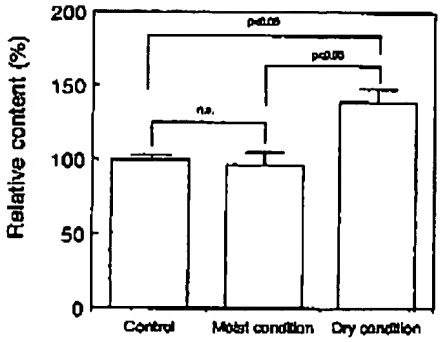

Fig. 6. The level of anti-DG I-reactive protein in SC from animals kept in the dry condition for 3 days was higher than that of animals kept in normal conditions ($n=4$, mean ± S.E.M.).

hyperproliferation of the nucleated cell layer of the epidermis. Further, a decrease of desmosomal degradation was observed. Because in our present in vitro assay the enzyme activity in SC of the dry group mice was not altered, the contents of the enzyme might be similar in both the dry and moist groups. On the other hand, we found that the water content of SC was decreased on and after day 1 in the mice which were kept in the dry condition. We interpret these in vitro and in vivo data as indicating that the dry condition makes the enzyme less active, and as a result of this, desmosomal degradation in the intact mouse SC was perturbed and abnormal scaling occurred without alteration of the metabolism in the nucleated cell layer. In the moist condition, the thickness of SC was unchanged (Fig. 4(b)) and the content of desmoglein I in SC was also unchanged (Fig. 6). These results suggest that the desquamation of SC were not changed in the moist condition as a whole. However, the number of scales on the backs of mice decreased on and after day 1. We measured the water content of SC in the moist condition and it increased on and after day 1. These results suggested that the SC surface desquamation accelerated by high water content, and as a result, visible scales disappeared earlier than in the animals which were kept in normal conditions.

Table 1
Changes of enzyme activity related to desquamation in dry condition

Time (day)	Enzyme activity (nmol released AMC/ml per mg SC)		
	Mean	S.E.M.	Significance versus day 0
0	22.49	6.10	
1	23.90	4.50	n.s.
2	25.43	7.29	n.s.
3	22.57	3.77	n.s.
4	23.74	4.46	n.s.
7	24.40	6.23	n.s.

Our study suggests that a dry environment has a rather rapid effect on normal skin in vivo and induces scaliness by impairing desquamation in SC. The SC water content seems to be a crucial factor in the regulation of SC desquamation.

More severe symptoms are seen in aged people in winter [11,19], and the shear wave technique showed that aged skin has a lower water content than that of younger skin [20]. Therefore, dry conditions might have a much more severe effect on the aged skin. The dry skin model presented here could be useful in studying the mechanism of skin problems induced by low humidity.

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The repair of impaired epidermal barrier function in rats by the cutaneous application of linoleic acid

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SUMMARY

Epidermal barrier function in rats was experimentally impaired by two separate means, namely, by rendering the animals deficient in essential fatty acids and by evoking a primary cutaneous irritant response by treating with a solution of sodium laurate. Impaired barrier function was manifested by a greatly increased rate of transepidermal water loss. Application to the skin of sunflower seed oil, which is rich in linoleic acid, rapidly restored to normal the abnormally high rates of transepidermal water loss in both experimental cases, and it was shown with the essential fatty acid-deficient rats that there was a concomitant incorporation of linoleic acid of the sunflower seed oil into epidermal lipids. Cutaneous application of olive oil, which is low in linoleic acid but rich in the non-essential oleic acid, did not influence epidermal barrier function. A close relationship of barrier function and essential fatty acids is indicated.

INTRODUCTION

The skin is a barrier between the living organism and its environment, and this barrier function resides in the stratum corneum (Blank, 1965). As well as being a barrier to the percutaneous entry of materials into the body, the skin also regulates the loss of body fluids; indeed, the rate of transepidermal water loss is a convenient parameter for expressing barrier function. In some skin diseases, in which the normal architecture of the stratum is altered, impaired barrier function occurs, with, in particular, increased transepidermal water loss. For example, in psoriasis the rate of transepidermal water loss is greatly increased compared with that of normal skin (Grice & Bentley, 1967). Similarly in the experimentally induced condition of essential fatty acid deficiency in rats, which is characterized by several changes in epidermal structure (Basnayake & Sinclair, 1956; Menton, 1968), the rate of transepidermal water loss is abnormally high. Also primary irritation of animal skin may cause cutaneous water permeability to be increased (Grasso & Lansdown, 1972), and structural changes may also be seen in the epidermis (Prottey, Hartop & Ferguson, 1973). These examples emphasize the close relationship of normal barrier properties of the skin to normal structural integrity of the epidermis.

In essential fatty acid-deficient rats the abnormally high rate of transepidermal water loss may be lowered to normal levels by the re-introduction of linoleic acid to the diet, when the cutaneous lesions

also disappear (Basnayake & Sinclair, 1956), suggesting a link between essential fatty acids and barrier function. We have recently shown (Press, Hartop & Prottey, 1974; Prottey, Hartop & Press, 1975) that essential fatty acid deficiency in man may be corrected by the cutaneous application of essential fatty acid (as sunflower seed oil, which contains 62% linoleic acid), which emphasizes the efficacy of the cutaneous route for introducing essential fatty acids into the body. In this paper, we have further examined the value of cutaneous application of sunflower seed oil, but in the context of repairing impaired barrier function. As models of increased cutaneous water diffusion we have chosen the EFA-deficient rat and the sodium laurate-irritated rat. The effect upon their impaired cutaneous barrier function by cutaneously applied sunflower seed oil is described.

MATERIALS AND METHODS

Treatment of essential fatty acid-deficient rats

Groups of male littermate rats of the Colworth-Wistar strain were weaned at 3 weeks onto a purified diet and an essential fatty acid-deficient diet. The purified diet consisted of 72·83% sucrose, 20·0% Fisons Genatosan (casein), 5·0% Jones-Foster salt mixture, 0·0468% water-soluble vitamins, 0·1% choline chloride and 2·17% sunflower seed oil. In the essential fatty acid-deficient diet the sunflower seed oil was replaced with 2·17% hardened coconut oil. The linoleic acid content of the sunflower seed oil was 62%, while the hardened coconut oil contained 2·0% unsaturated fatty acids but no essential fatty acids. Two groups of rats were fed each diet for 9 weeks and for 19 weeks respectively. EFA-deficiency was characterized by scaliness of the feet and tail, increased transepidermal water loss, and the appearance of 5, 8, 11-eicosatrienoic acid in the serum lipids, with the concomitant reduction of linoleic and arachidonic acids (Holman, 1968). By 9 weeks on the deficient diets the animals were all EFA-deficient. In the first experiment rats were studied at 8 and 18 weeks on the diet, in the second experiment, at 12 weeks.

In the first experiment, after 8 and 18 weeks on the control and experimental diets, a small area of approximately 1 cm^2 was clipped on the interscapular skin of each rat. The groups of control and EFA-deficient rats were each sub-divided into two groups, one of which had no cutaneous treatment while the other received 0·1 ml of sunflower seed oil to the clipped skin twice daily for 7 days. This was equivalent to approximately 120 mg linoleic acid per day.

In order to prevent ingestion of the oil, all the animals were fitted with a headpiece of rigid nylon around the neck without cutting into the skin. The thin conical walls of the neckpiece extended over the head of the rat. The open end allowed the rat to eat and drink but prevented it from licking its paws or any part of its back. During these 7 days the eyes, nose and penis of the rats were cleaned to avoid possible infection. Transepidermal water-loss measurements were made immediately before and after the 7 days' application of sunflower seed oil.

In the second experiment, animals were taken after 12 weeks on the deficient diet. The dorsal hair was removed by clipping, and then to one group of six animals 0·1 ml of a solution of 167 mg/ml sunflower seed oil in ether was applied to the shaved skin daily for 5 days. Assuming that sunflower seed oil contains about 60% by weight linoleic acid, this is equivalent to the application of 10 mg linoleic acid to the skin daily. To a second group of six animals a similar volume of an ether solution of 133 mg/ml olive oil was applied. Olive oil contains approximately 75% by weight oleic acid, thus the amount applied cutaneously per day was 10 mg oleic acid. A third group, but of normal rats fed on the control diet for 12 weeks, received 10 mg linoleic acid cutaneously, as above, to serve as controls to the EFA-deficient animals. In this second experiment the nylon head pieces were not fitted to the animals. Transepidermal water loss was measured daily immediately before application of oil for 6 successive days.

Treatment of rats with sodium laurate

Female weanling rats (approximately 50–80 g) fed water and spital diet *ad libitum* were used exclusively for this experiment. At the beginning of the experiment each animal was closely clipped dorsally with electric hair clippers.

The rate of transepidermal water loss was determined on the clipped area of each rat prior to any treatment. Each animal was washed with 0·25 M sodium laurate solution for a period of 30 min, as previously described (Prottey & Hartop, 1973). After a period of 24 h had elapsed, to allow the irritation response to develop, the water loss was determined again. Individual responses to treatment were varied, some animals exhibiting very high rates of water loss, whereas others had lower, yet still above normal, rates relative to control values. The animals were divided into three groups so that each group contained examples of both high and low rates of water loss after irritation. One group had 0·1 ml of undiluted sunflower seed oil rubbed onto the irritated area, a second group received 0·1 ml undiluted olive oil (the treated control group), and a third group were left untreated, serving as untreated controls to both treated groups. Oil was applied to the skin immediately after the second determination of water loss. The rate of transepidermal water loss was determined for a third time on each animal 24 h after receiving the oil.

Determination of transepidermal water loss

The method used was originally developed by Thiele & Schutter (1963); a stream of dry nitrogen was passed over the skin by means of a flow cell and its increased moisture content was measured with a Meeco electrolytic moisture analyser (Manufacturers Engineering and Equipment Corporation, Warrington, Pennsylvania, U.S.A.). The time required to make readings was approximately 30 min, during which the rats were immobilized with Nembutal (75 mg per kg intraperitoneally) and placed in a controlled temperature chamber at 34·5 ± 0·5 °C. The gas flow cell, which was fitted with a thermistor to measure skin temperature, was strapped to the clipped dorsal skin with Velcro tape (Selectus Ltd., Biddulph, Staffordshire) and transepidermal water loss and skin temperature were recorded simultaneously on a twin channel recorder before and after application of oil.

Extraction and analysis of epidermal lipids

Rat epidermis was separated by heat (Baumberger, Suntzeff & Cowdrey, 1942), the epidermal lipids purified by thin-layer chromatography and fatty acid compositions were determined by gas–liquid chromatography (Prottrey, Hartop & Ferguson, 1972).

RESULTS

Transepidermal water loss in essential fatty acid-deficient rats

The increase in transepidermal water loss in animals maintained on an essential fatty acid-deficient diet is illustrated in Table 1. Rats on the control diet showed low rates of transepidermal water loss at 9 and 19 weeks, and the cutaneous application of sunflower seed oil for 1 week after 8 and 18 weeks respectively did not greatly alter these. Rats maintained on the deficient diet, however, showed a significant increase ($P = 0{\cdot}001$) in water diffusion compared with controls after 9 weeks; after 19 weeks the increase was greater still. After cutaneous application of sunflower seed oil for 7 days at 8 weeks there was a significant reduction in the transepidermal water loss of the EFA-deficient animals ($P = 0{\cdot}001$).

In the two deficient animals treated for 7 days, after 18 weeks there was also a marked reduction in water loss. However, in both treatment cases (that is, after 9 weeks and 19 weeks) the observed values

TABLE 1. EFA deficiency and transepidermal water loss

Diet	Treatment of skin	Transepidermal water loss (mg/cm²/h) after weeks on diet 9*	19†
Control	Nil	0·26 ± 0·09 (3)	0·24, 0·34
	SSO	0·20 ± 0·03 (3)	0·39, 0·30
EFA deficient	Nil	3·07 ± 0·90 (3)	4·36, 4·12
	SSO	1·34 ± 0·52 (3)	1·37, 1·58

EFA, essential fatty acid; SSO, sunflower seed oil.
* Results are mean data ± s.d. for number of animals in parentheses.
† Results are from individual animals.

of transepidermal water loss had not attained the very low level shown by control animals on a normal diet.

Epidermal lipid fatty acid composition in essential fatty acid-deficient rats

The application of sunflower seed oil to the skin of normal rats on the control diet for 9 weeks (Table 2) caused an increase in the proportion of linoleic acid in the free fatty acid fraction of the epidermal

TABLE 2. Distribution of fatty acids in separated lipids of epidermis of control and EFA-deficient rats at 9 weeks

Diet (9 weeks)	Cutaneous treatment at 8 weeks	Lipid fraction	Fatty acid (%) 16:1	18:1	18:2	20:3 (ω-9)	20:3 (ω-6)	20:4
Control	Nil	PL	7·0 ± 1·9	15·9 ± 1·9	9·4 ± 1·7	T	0·5 ± 0·9	6·2 ± 0·3
	SSO		6·6 ± 2·5	18·6 ± 1·0	12·6 ± 2·5	T	1·6 ± 2·5	5·4 ± 3·0
	Nil	SE	7·2 ± 1·7	15·2 ± 1·2	2·2 ± 1·0	T	0·4 ± 0·6	1·2 ± 0·0
	SSO		5·1 ± 1·3	14·2 ± 1·2	5·6 ± 0·6	T	0·3 ± 0·3	1·0 ± 0·6
	Nil	FFA	3·6 ± 0·8	14·3 ± 2·8	5·3 ± 1·6	T	1·2 ± 1·2	1·8 ± 0·7
	SSO		2·7 ± 0·2	18·8 ± 6·6	20·4 ± 15·8	0·3 ± 0·5	0·4 ± 0·7	1·7 ± 0·3
	Nil	TG	4·0 ± 0·9	12·6 ± 3·6	4·6 ± 1·5	T	0·6 ± 0·7	2·6 ± 1·9
	SSO		6·0 ± 2·4	14·8 ± 3·8	3·7 ± 1·5	T	1·1 ± 0·8	2·3 ± 2·4
EFA-deficient	Nil	PL	9·2 ± 4·6	25·7 ± 4·3	1·0 ± 1·2	4·1 ± 1·8	0·3 ± 0·2	1·2 ± 1·3
	SSO		8·3 ± 3·2	21·2 ± 4·4	8·4 ± 4·1	4·4 ± 2·3	1·0 ± 0·5	1·5 ± 0·9
	Nil	SE	8·3 ± 2·3	16·4 ± 4·3	0·5 ± 0·6	T	0·4 ± 0·4	1·8 ± 0·5
	SSO		7·3 ± 0·7	21·1 ± 2·0	1·3 ± 0·2	0·4 ± 0·7	0·6 ± 0·7	2·2 ± 2·1
	Nil	FFA	6·6 ± 2·1	16·7 ± 3·3	2·2 ± 1·3	2·1 ± 0·6	1·7 ± 0·8	2·0 ± 0·9
	SSO		4·5 ± 3·3	25·7 ± 5·7	22·2 ± 15·7	0·7 ± 0·6	0·8 ± 1·1	0·6 ± 1·0
	Nil	TG	7·4 ± 1·5	20·6 ± 11·7	3·0 ± 1·4	0·7 ± 1·1	0·6 ± 1·0	1·7 ± 1·3
	SSO		4·2 ± 1·8	20·4 ± 7·8	15·4 ± 6·9	2·3 ± 2·2	0·6 ± 1·0	1·6 ± 1·4

SSO, sunflower seed oil; PL, phospholipids; SE, sterol esters; FFA, free fatty acids; TG, triglycerides.
The results are the means of three animals ± s.d.
T, trace (< 0·1%).

TABLE 3. Distribution of fatty acids in separated lipids of epidermis of control and EFA-deficient rats at 19 weeks

Diet (19 weeks)	Topical treatment at 18 weeks	Lipid fraction	Fatty acid (%) 16:1	18:1	18:2	20:3 (ω-9)	20:3 (ω-6)	20:4
Control	Nil	PL	6·7, 7·9	14·6, 16·5	8·1, 10·2	T, T.	1·1, 2·2	5·8, 5·0
	SSO		5·8, 6·0	15·5, 16·1	10·8, 7·9	T, T	0·5, T	6·6, 3·5
	Nil	SE	5·4, 5·6	9·1, 15·6	2·4, 1·9	T, T	T, 2·8	2·7, 0·6
	SSO		5·6, 4·4	12·5, 14·4	2·2, 4·2	1·0, T	0·6, 5·1	1·5, 1·7
	Nil	FFA	2·9, 3·0	11·0, 14·3	4·5, 3·2	T, T	3·6, 0·6	8·8, 0·8
	SSO		4·0, 4·6	10·1, 19·9	6·8, 16·5	0·5, T	1·5, 4·2	2·9, 1·0
	Nil	TG	4·3, 4·0	18·5, 16·5	7·0, 7·3	T, T	T, 1·0	1·0, 0·9
	SSO		2·7, 4·4	18·9, 23·8	14·7, 8·1	T, T	T, T	1·7, 0·2
EFA-deficient	Nil	PL	10·5, 9·5	19·1, 19·6	0·9, 1·1	2·5, 11·4	T, T	0·9, 1·9
	SSO		9·1, 10·1	21·7, 24·2	5·9, 6·7	3·3, 3·4	T, T	1·2, 1·0
	Nil	SE	9·3, 7·8	21·4, 22·0	0·9, 0·9	0·3, 0·7	T, T	0·5, 1·0
	SSO		5·2, 4·2	17·8, 14·7	1·0, 3·2	T, 7·2	1·3, T	3·5, 1·1
	Nil	FFA	7·6, 8·0	22·2, 30·0	1·4, 2·8	4·9, 3·9	0·9, T	1·8, 2·2
	SSO		5·0, 4·2	17·0, 18·9	4·4, 12·9	1·5, 1·9	0·3, 1·1	2·3, 1·2
	Nil	TG	6·6, 9·9	16·1, 32·5	0·5, 3·5	T, 4·5	T, T	1·1, 3·6
	SSO		3·7, 2·7	22·7, 18·7	8·7, 7·6	0·2, 1·3	T, T	3·2, 2·0

SSO, sunflower seed oil; PL, phospholipids; SE, sterol esters; FFA, free fatty acids; TG, triglycerides.
The results are the individual values from two animals.
T, trace (<0·1%).

lipids, but no marked changes were seen in the amounts of this in the other lipid fractions. Rats maintained on the essential fatty acid-deficient diet for 9 weeks (Table 2) showed increased oleic acid in the phospholipid and triglyceride fractions and in 5, 8, 11-eicosatrienoic acid (ω-9) in the phospholipids, together with a decrease in the linoleic and arachidonic acids of the phospholipids. The 5, 8,

TABLE 4. Comparison of the effects of cutaneously applied linoleic and oleic acid on lowering transepidermal water loss of EFA-deficient rats

Animals		Transepidermal water loss (mg/cm²/h) Before application	After application (days) 1	2	3	4	5
EFA-deficient (6)	Linoleic acid (as sunflower seed oil)	2·33 ± 0·56 (12)	2·57 ± 0·42	2·64 ± 0·65	2·17 ± 0·70	1·41 ± 0·60	1·10 ± 0·38
EFA-deficient (6)	Oleic acid (as olive oil)		2·18 ± 0·43	2·22 ± 0·69	3·06 ± 1·31	2·76 ± 0·67	1·99 ± 0·78
Normal controls	Linoleic acid	0·48 ± 0·12 (6)	0·46 ± 0·10	0·48 ± 0·15	0·36 ± 0·15	0·42 ± 0·10	0·36 ± 0·18

Values in parentheses represent number of animals of each group, from which means and standard deviations were calculated.

11-eicosatrienoic acid, which is absent in normal tissues, is the biochemical 'hallmark' of essential fatty acid deficiency (Fulco & Meads, 1959). There was no appreciable change in the 8, 11, 14-eicosatrienoic acid (ω-6) of any of the lipid classes. When the deficient animals were treated with sunflower seed oil, however, the linoleic acid of the phospholipid fraction increased to virtually the control level, but no appreciable increase in the properties of the eicosatrienoic (ω-6) and arachidonic acids in any of the lipid fractions was seen after treatment. These latter two acids are metabolic derivatives of linoleic acid.

After 19 weeks on the control diet (Table 3) the fatty acid distribution of the epidermis was very similar to that of the 9 week controls, and the effect of cutaneous application of sunflower seed oil was to increase the proportion of linoleic acid in the free fatty acid and triglyceride fractions. After 19 weeks on the deficient diet (Table 3) the main changes were an increase in the proportions of oleic and 5, 8, 11-eicosatrienoic (ω-9) acids and a slightly less marked increase in palmitoleic acid in all lipid fractions, and the three essential fatty acids, linoleic; 8, 11, 14-eicosatrienoic (ω-6) and arachidonic acids were all low, compared to control animals. Application of sunflower seed oil for 1 week to the deficient rats at this time caused increases in linoleic acid in free fatty acids, phospholipids and triglycerides, but there were no changes in the 8, 11, 14-eicosatrienoic (ω-6) or arachidonic acids.

Rapidity of the response of lowered transepidermal water loss to cutaneous application

In a second experiment the effect of linoleic acid (as sunflower seed oil) on EFA-deficient rat skin during the first few days of treatment was investigated on a group of six animals. A separate group of six animals received olive oil (rich in oleic acid), and normal (non-deficient) rats treated with sunflower seed oil served as controls. Table 4 gives the calculated daily mean values of transepidermal water loss for the 3 groups. The standard deviations of these means can be seen to be quite large in some cases, indicating that there was a wide spread of individual values of transepidermal water loss within the various groups. In order to test the statistical significance of the results, the daily changes in transepidermal water loss as a result of oil application were examined by Student's 't'-test of the differences between means of the paired observations before treatment and day by day after treatment. When each mean daily observation of transepidermal water loss was compared with its corresponding pre-treatment value, with sunflower seed oil-treated EFA-deficient rats there was a marked reduction in transepidermal water loss after 4 days which continued into the 5th day; these reductions were significant at the $P = 0{\cdot}01$ level of significance. With the olive oil-treated animals, although the daily mean values of water loss fluctuated, none was significantly different from the pre-treatment values. Normal control animals lost very small amounts of water, which did not alter during 5 days of application of linoleic acid. In this experiment the animals received approximately 1/6th of the amount of linoleic acid administered to those of the first experiment. None the less, transepidermal water loss was markedly reduced by cutaneous application after 4 days.

Effect of sodium laurate on transepidermal water loss in normal rats

Prior to application of sodium laurate the rate of transepidermal water loss was determined in each of the eighteen rats, and the mean value was calculated as $0{\cdot}096$ mg/cm^2/h, with a standard deviation of $\pm 0{\cdot}047$. These animals were weanlings (3–4 weeks old) and the mean value of water diffusion was much less than that seen with the control animals of Tables 1 and 4. As the latter were between 3 and 6 months of age, with different epidermal thickness and hair growth patterns, such differences are not unexpected.

In every case, 24 h after application of sodium laurate solution to the skin the rate of transepidermal water loss was seen to have increased. Most of the increased values were in the range 1–3 mg/cm^2/h, but three animals in particular had very high readings (the highest of which was 9·299 mg/cm^2/h)

TABLE 5. Effect of cutaneous application of sunflower seed oil and olive oil upon the recovery of transepidermal water loss after soap irritation

Treatment group	Mean water diffusion rate ± s.d. (mg/cm²/h)		
	Before irritation	After soap irritation*	After topical application
No application	0·091 ± 0·036 (6)	2·959 ± 3·201 (6)	1·777 ± 0·929 (6)
Sunflower seed oil	0·086 ± 0·051 (6)	2·411 ± 2·889 (6)	0·468 ± 0·155 (6)
Olive oil	0·110 ± 0·056 (6)	2·680 ± 2·595 (6)	2·279 ± 0·655 (6)

* Each animal was treated with 0·25 M sodium laurate for 30 min during 1 day.
The figure in parentheses denotes the number of experimental observations, from which the statistics were derived.

indicative of strong responses to sodium laurate application. The calculated mean increased value of transepidermal water loss for the eighteen animals was 2·68 mg/cm²/h, but, as the range of individual values was wide, the standard deviation of this mean was ±2·74 mg/cm²/h. None-the-less, a Student's 't'-test of the difference between the pre- and post-treatment means of paired observations was significant at $P = 0{\cdot}01$. The irritation response of the skin of these animals as a result of sodium laurate application was also varied, ranging from mild erythema to drying and cracking of the stratum corneum. No accurate subjective assessment of the degree of irritation was performed (Frottey, Hartop & Fergusson, 1973) and so semiquantitative comparison of transepidermal water loss and skin irritation could not be made.

Effect of cutaneous application of vegetable oils on water diffusion of sodium laurate-treated rats

After determination of the rate of transepidermal water loss in the irritated animals, they were divided into three groups, such that each group contained animals of high and low diffusion rates. Those in the first group received no further treatment, in the second group, sunflower seed oil, and in the third group olive oil. After a further 24 h water diffusion rates were measured and the results are shown in Table 5. All animals which received cutaneous sunflower seed oil exhibited lower values of transepidermal water loss (mean value of 0·408 mg/cm²/h), but a Student's paired 't'-test of the significance of these changes compared with after sodium laurate application alone proved to be non-significant at the 95% level of significance (presumably due to the wide spread of individual values, reflected by the standard deviations). In both the group of rats receiving olive oil and the untreated controls, the mean values of transepidermal water loss were not markedly lower than after sodium laurate application. In the group of rats receiving sunflower seed oil compared with both that group receiving olive oil and that group remaining untreated, transepidermal water loss was significantly lower ($P = 0{\cdot}01$), suggesting that it is the linoleic acid predominant in this oil which is responsible for repairing the abnormal barrier function.

DISCUSSION

We have shown that cutaneous application of linoleic acid, in the form of a triglyceride ester as sunflower seed oil, restores to normal the abnormally high rate of transepidermal water loss in two experimentally individual conditions of rat skin, namely, essential fatty acid deficiency and primary irritation by sodium laurate. Restoration to normal of high rates of water diffusion in essential fatty acid-deficient rats has been previously demonstrated by re-introduction to the diet of essential fat

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(Basnayake & Sinclair, 1956). There are earlier references to the cutaneous application of linoleic acid to essential fatty acid-deficient rats: in 1936 Shepherd & Linn described how application of 'Vitamin F' (as linoleic acid was described) to the tail of an essential fatty acid-deficient rat cured the scaliness which characterized the condition; Grandel (1938) later extended these studies. We have recently demonstrated restoration of the cutaneous manifestations of EFA-deficiency in man by this means (Prottey *et al.*, 1975). Beneficial properties of cutaneously applied ethyl linoleate have also been shown recently by Jelenko, Wheeler & Scott (1972) for burned skin, in which the abnormally high rate of water transmission was reduced by up to 50%. Therefore, our finding here that impaired skin barrier function as a result of primary irritation may be restored by cutaneous sunflower seed oil is not unexpected.

As well as the similar results of sunflower seed oil application in both experimental models of impaired barrier function described here, there are also other similarities. It has been amply described (Williamson, 1941; Basnayake & Sinclair, 1956; Menton, 1968) that the skin in EFA-deficiency is hyperplastic and hyperkeratotic with a consequential thickening of the stratum corneum. We have shown (Prottey & Hartop, 1973; Prottey *et al.*, 1973) that rat skin treated with sodium laurate solution also exhibits morphological changes which have some similarities to essential fatty acid deficiency, such as hyperplasia, stratum corneum thickening and increased mitotic activity. Also, in EFA-deficiency in the rat, deranged lipid metabolism (Ziboh & Hsia, 1972) and accumulation of fat in the stratum corneum (Kingery & Kellum, 1965) have been reported. Similarly, it is known that following surfactant irritation of rat skin the epidermis synthesises more lipid (Prottey & Hartop, 1973), and lipid droplets may be observed in the stratum corneum (Tovell *et al.*, 1974).

Despite these similarities, it cannot be assumed that surfactant-irritated rat skin may be equated with detergent-treated skin. Elevation of the rate of transepidermal water loss is a general phenomenon that has been described in animals and man alike. Sweeney & Downing (1970) showed that solvent treatment of mouse skin increased the rate of water diffusion; tape stripped human skin has a similarly raised rate (Spruit & Malten, 1968; Eriksson & Lanke, 1971) and in certain skin diseases transepidermal water loss is increased (Grice & Bentley, 1967; Rajka, 1974). None the less, the beneficial action of sunflower seed oil in repairing barrier function may indicate an important function of linoleic acid; Onken & Moyer (1963) demonstrated the involvement of lipids in barrier function. Tables 2 and 3 indicate that cutaneous application of sunflower seed oil to EFA-deficient skin resulted in its incorporation into phospholipids, and we have similar (unreported) information for detergent-irritated skin so treated. Linoleic acid has been identified in human epidermal phospholipids (Vroman, Nemecek & Hsia, 1969).

It was found that the effect of sunflower seed oil on sodium laurate-irritated skin was rapid reduction in transepidermal water loss within 24 h, whereas on EFA-deficient skin a reduction in water loss of a similar magnitude required 4 to 5 days, and so the precise mechanism of the barrier repair may be different in these two instances. A physical occlusive effect may be discounted as olive oil did not effect such a reduction in water loss. Indeed, in separate studies (to be reported) we have examined other oils, and the beneficial action on impaired barrier function seems to be confined to certain polyunsaturated fatty acids of the ω-6 configuration; oils containing non-essential fatty acids have no effect.

Recently, Ziboh & Hsia (1972) have described how the cutaneous application of prostaglandin E_2 to the skin of essential fatty acid-deficient rats cleared the scaly lesions, and also reversed the abnormal sterol ester metabolism. Thus it may be speculated that the repair of barrier function we have described above is, in fact, due to conversion of the cutaneously applied linoleic acid, via arachidonic acid, into prostaglandin E_2, instead of (or in addition to) its incorporation into phospholipids which constitute the barrier function of the skin. This is currently under investigation.

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